

MICROSTRUCTURAL CHANGES AND MICRONUTRIENT BIOACCESSIBILITY IN
SOFT-SOLID FOODS, USING A MODEL FRESH CHEESE AND *IN VITRO* DIGESTION

BY

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THESIS

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Abstract

Processed food products are complex matrices with many components interacting below 100 μm . These interactions will dictate the characteristics of product from a physical, chemical and nutritional point of view. The increasing demand for food products with health benefits and functional properties encourages the use of material and physical science to understand how food components interact at the mesoscale. The fundamental understanding of this interaction and its relationship will open new windows for food engineering and innovation to create structures with specific functionalities especially for nutritional availability in processed food matrices.

The overall objective of this study was to evaluate the structural characteristics of a soft solid matrix created by variations in formulation and processing parameters, and its relationship with the bioaccessibility of liposoluble vitamins through *in vitro* digestion. There were two specific aims to accomplish the overall objective. The first aim was to evaluate the structure and microstructure of the soft solid matrix, using a model fresh cheese processed at a various protein to fat ratios and homogenization pressures by means of textural, rheological and image analysis. The second aim was to evaluate the bioaccessibility of liposoluble vitamins through *in vitro* digestion by fortifying a soft solid matrix with vitamin D₃, using a model fresh cheese processed at a various protein to fat ratios and homogenization pressures.

To accomplish these objectives, a model fresh cheese was prepared at various protein to fat ratios and homogenization pressures. Particle size of the cheese milk emulsion was analyzed by laser diffraction. Structural parameters were measured by large and small deformations using Texture Profile Analysis (TPA) and Small Amplitude Oscillatory Shear (SAOS) rheology. Microstructure, specifically pore size, and porosity was analyzed by Environmental Scanning Electron Microscopy (ESEM) micrographs. Three experimental units (containing the lowest and

highest protein to fat ratios and lowest and highest homogenization pressures) were selected for vitamin D₃ bioaccessibility analysis using an *in vitro* digestion model.

Protein to fat ratio and homogenization pressure significantly affected the particle size of the cheese milk emulsion. At higher protein to fat ratio and homogenization pressure particle size was reduced significantly. Similarly, at a higher protein to fat ratios and homogenization pressures samples were significantly harder and cohesive at large deformations, however, no clear trend was seen from small deformations nor porosity. On the other hand, bioaccessibility of vitamin D₃ decreased at higher protein to fat ratios and homogenization pressures suggesting higher protein-protein interactions during the processing of the model cheese and perhaps lower protein hydrolysis during the *in vitro* digestion.

The study of complex food matrices needs further investigation since many factors regarding constituents as well as processing, are interacting together. Critical interactions between food components during processing are directly related to the physical characteristics of food products as well as their behavior during digestion. Therefore, understanding the food matrix and its behavior during processing and digestion is essential for engineering food structure with specific functionality.

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Chapter 1. Introduction

1.1 Motivation

Food product innovation is related to the utilization of diverse materials and ingredients, or to the application of new technologies in processing and packaging. The increasing demand of consumers to access healthier products with specific functionalities and also to obtain more information regarding the ingredients and nutritional characteristics of food products is currently the driving force for research and development (Parada and Aguilera 2007). At the same time, the introduction of new techniques of analysis from material science and physics into food science has led to study food products, specifically food components and matrices from a microstructural perspective (Aguilera and Stanley 1999), where the physical state and interactions of several components can be evaluated at the microscale ($< 100 \mu\text{m}$) (Aguilera 2005; Mezzenga and others 2005). Nutritional and functional properties of a food product also might be studied from a microstructural perspective considering the possible linkage between food structure, and availability of nutrients (Le Feunteun and others 2014). Since processed food products are very complex and non-equilibrated systems, the study of microstructure and its relationship with nutrient availability can be very extensive. Despite the fact that some studies have shown a linkage between food matrix and availability of nutrients (Panozzo and others 2013; Gallier and others 2014; Rodríguez-Roque and others 2015), there is few information related to highly processed food matrix and the effects of composition and processing parameters on the conformation of diverse structures and its relationship with nutrient availability. . Understanding the microstructure of processed foods and the effects of composition and processing in nutrient availability would allow to engineered new products with diverse functional characteristics. The interest of this study is to evaluate how micronutrient

bioaccessibility might be affected by the food microstructure in a processed soft solid matrix; a structural characteristic found in many commercially available food products. In order to assess this relationship, a model fresh cheese has been selected as an example of a soft solid matrix, and the variables composition (protein to fat ratio) and processing (homogenization pressure) will be used at different levels to evaluate the structural and microstructural characteristics and subsequent bioaccessibility of vitamin D₃ by *in vitro* digestion. The processing variable that will be utilized is microfluidization, which will be used indistinctly as homogenization, in the same way, the formulation parameter to be utilized is protein to fat ratio, and will be indicated as P/F.

1.2 Overall Hypothesis and Goal

The hypothesis of this research is that increasing protein to fat ratios and homogenization pressures in a model fresh cheese creates a structural network with smaller pores and particle sizes, resulting in a more rigid and compact structure with an increased fat globule surface area, and thus an enhanced bioaccessibility of vitamin D₃. The goal is to evaluate the structure created by variations in formulation and homogenization pressures of a model fresh cheese and its relationship with vitamin D₃ bioaccessibility.

1.3 Specific Objectives

The specific objectives of this research:

- 1) Evaluate the structure and microstructure of a model fresh cheese processed at various protein to fat ratios (P/F) and homogenization pressures by means of textural, rheological and imaging analysis.

The hypothesis of this objective is that increasing protein to fat ratio and homogenization pressures will decrease the particle size of the cheese milk emulsion resulting in a more rigid and

elastic model fresh cheese matrix with a smaller pore size. Particle size of the cheese milk emulsions was measured by laser diffraction; textural properties of the model fresh cheeses were measured by large and small deformations using Texture Profile Analysis (TPA) and Small Amplitude Oscillatory Shear (SAOS) rheometry. The microstructure was analyzed by Environmental Scanning Electron Microscopy (ESEM) where porosity and pore size was determined for each treatment.

- 2) Evaluate the bioaccessibility of vitamin D₃ from a fortified model fresh cheese processed at various protein to fat ratios (P/F) and homogenization pressures.

The hypothesis of this objective is that increasing protein to fat ratios and homogenization pressures increases the interfacial surface area of fat particles in the matrix of a model fresh cheese which makes vitamin D₃ more bioaccessible after *in vitro* digestion. Vitamin D₃ was extracted from the cheese matrix using a saponification step and liquid extraction with solvents; bioaccessibility was assessed by a two phase simulated *in-vitro* digestion.

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Chapter 2. Literature Review

2.1 Introduction

Innovation in food products has always been guided by the consumers' needs and expectations. In recent years the linkage between food consumption and the prevalence of non-communicable diseases has become more evident, and that is one of the main reasons consumers are demanding healthier products with functional characteristics that provides taste, satisfaction and wellness (Parada and Aguilera 2007). In the race for creating functional products with positive health connotations, food scientists are combining a series of technologies adapted from other fields such as chemistry, physics, and mechanical engineering to study, create and deliver these types of products in a large scale production. The application of image analysis to food science has allowed to study food structure at a micro and nano-level ($<100\mu\text{m}$) (Aguilera and others 2000) and thus is easier to understand how the components interact in the food matrix at the macro-scale level., There is an increased interest to elucidate how these interactions are modified under certain processing and formulation conditions, and if there is a linkage between structure and nutrient availability. Since food is a very complex and non-equilibrated matrix, studies using model food systems have been conducted to assess structure at the micro and nano scales. The use of hydrocolloids, as well as proteins, represents an appropriate avenue to recreate food structures due to their versatility on structure formation (Aguilera and Stanley 1999; Stokes 2012; Zuniga and Troncoso 2012; Fernández Farrés and others 2014) especially for mimicking soft solid matrices, which are commonly found in several food products.

Understanding food microstructure and its relationship with nutrient availability will open new opportunities for product development with specific functional characteristics as well as new opportunities for providing nutritional benefits to the consumer.

2.2 Food microstructure and bioaccessibility of nutrients

Food microstructure refers to all food components that interact in the food matrix at the molecular and mesoscale levels below 100 μm , in which the length scale of the most dominant component will determine the macrostructural characteristics (Ubbink and others 2008). Aguilera (2005, 2012) presented approximate sizes of some food components to elucidate their structural dimension (Figure 2.1). Foods can be studied and engineered at these length scales where all components (including nutrients) interact. The nutrient composition of food products is well documented. However the amount of nutrient that is absorbed during digestion is somewhat uncertain since it may vary according to processing conditions or the same interaction between components (Parada and Aguilera 2007); by saying this, it can be inferred that there is a relationship between food matrix, nutrient disposition within the matrix, bioaccessibility, and subsequent bioavailability. The term bioaccessibility can be defined as the fraction of a compound that is released from the food matrix in the gastrointestinal (GI) tract and becomes available for intestinal absorption (Fernández-García and others 2009). The proportion that is absorbed and utilized by the body on its biological functions is called bioavailability (Fairweather-Tait and Southon 2003). These two concepts need to be further studied to establish the interactions between food matrix and nutrient absorption (Zuniga and Troncoso 2012).

There are several studies that relate the effects of processing on food microstructure and nutrient digestion and absorption. Some of them have been focused on the analysis of minimally processed matrices and others in more complex ones. Table 2.1 summarizes recent studies in which processing, formulation, and food matrix have demonstrated to play a significant role in

nutrient bioaccessibility or bioavailability. Bioaccessibility is usually evaluated by in vitro digestion, and bioaccessibility by in vivo models using human or animal subjects (Sensoy 2014a). In vitro digestion is conducted by simulating the digestion process using a combination of digestive enzymes under controlled conditions of pH, temperature, time and physical processes (shear or mixing) (Parada and Aguilera 2007). This method has the advantage of being safe and rapid compared to in vivo methods that required longer periods of time and strict regulations that allow sampling in human or animal subjects. On the other hand, in vivo methods provide direct bioaccessibility and bioavailability data. However in vivo studies need to be conducted carefully since other physiological variables might be included (Parada and Aguilera 2007; Sensoy 2014b).

2.3 Cheese as an example of soft solid matrix

Soft-solids/soft matter

Food is a complex and non-equilibrated system that can be organized and re-organized at different levels (Aguilera 2012). The complexity of food is the result of the interaction of its components at various length scales (macro to nano scale) which is key to defining its structural characteristics and functionality (Mezzenga and others 2005; Ubbink and others 2008). A wide variety of commercially available food products show a combination of solid-and-liquid like properties on their structure (Stokes 2012), this characteristic has been referred as “soft-solid” or “soft-matter” (Aguilera and Stanley 1999; Stokes and Frith 2008). Soft matter is defined as a complex system with a dispersed mesoscopic structure that might include gas bubbles, colloidal particles, emulsion droplets, amphiphiles or polymers (van der Sman 2012), that can be found in many high moisture processed food products such as jellies, jam, processed meats and dairy products (Aguilera and Stanley 1999; Stokes and Frith 2008).

The study of soft-solids is of great importance to food science, because of its versatility on developing food model systems that describe not only a wide variety of structures including colloids, polymers, emulsions and suspensions (Hirst 2013), but also their hierarchical assemblies at the mesoscale (microstructure) such as micelles, fibers, nano and micro emulsions, gels and crystals (Gunstone 2006; Lang and Liu 2016). In this regard, milk protein gelation have been intensively investigated due to its ability for imparting structure, texture, flavor and functionality to foods especially to dairy-based products (Remondetto and Subirade 2003; Van Vliet and others 2004).

Cheese is a good example of a combination of the mesoscale building blocks, where dispersed casein micelles are intentionally aggregated by acidification, or due to enzymatic action (Aguilera and Stanley 1999; Mezzenga and others 2005; Aguilera 2012); creating a micro-structural network in which solid fat globules and serum phase are entrapped forming a soft-solid state (van der Sman 2012).

Cheese microstructure affected by composition and processing parameters

As mentioned before, the mesoscale building blocks of cheese are arranged in a network structure composed of micellar milk proteins, fat globules, serum phase, minerals and vitamins (Figure 2.2 and 2.3). Milk proteins are divided into two major families, caseins and whey proteins (Kindstedt 2005). Caseins encompass about 80% from the total protein content, and it is formed by four different molecules (α_{s1} -, α_{s2} -, β and k-casein), this structures combined with calcium phosphate forms self-associated micelles (Dalgleish and Corredig 2012). The stability of casein micelle as a colloid is given by the organization of its protein molecules. k-Casein can be found in greatest quantities on the surface, which gives amphiphilic properties to the molecules

due to steric stabilization, and a protective capacity against precipitation (Holt and Horne 1996). On the other hand, 20% of the total milk proteins are formed by whey or serum proteins (β -lactoglobulin, α -lactalbumin, blood serum albumin and immunoglobulins) which generally will remain in the aqueous phase of the cheese once aggregation is performed (Fox and others 2000; Fox and McSweeney 2003). Meanwhile, lipids in milk are composed mostly by triglycerides (98%) and a combination of diglycerides, monoglycerides, fatty acids, phospholipids and sterols (2%) (Fox and McSweeney 2003). Besides these two major components (proteins and lipids), cheese and dairy products in general, are considered a good source of micronutrients.

Particularly, cheese is a good dietary source of calcium, magnesium, phosphorus, potassium and sodium (Reeker 1988) as well as small amounts of vitamins A, riboflavin, vitamin B12 and folate (O'Brien and O'Connor 2004). The concentration of micronutrients in cheese will be determined by the milk composition, type of cheese prepared and processing parameters (Dalglish and others 1996; Öste and others 1997; O'Brien and O'Connor 2004).

The structural conformation of cheese is considered to be a key factor for textural and functional characteristics (Joshi and others 2004). For example, variations in the ratio of the two main components of cheese (proteins and fat) can have an impact on texture, meltability, and flavor. Rogers and others (2009) demonstrated that low fat Cheddar cheeses differed from full fat, being the low fat cheeses more firm and springy, conversely the higher fat content cheeses showed to break down and deform at a lower force than low fat. Other authors explain this behavior stating that high fat contents cause a weakening of the structure by the moderate disruption of the casein matrix, which also depends on the fat globule size; fewer and smaller size fat globules might not disrupt the casein matrix since is expected they would be entrapped within (Everett 2007; Hickey and others 2015).

Homogenization pressure of cheese milk also can affect the structure. In a regular industrial process, milk is homogenized at pressures around 1500 – 2900 PSI (Pereda and others 2007) to prevent cream separation and maintain its stability during storage. In cheese milk, homogenization will disrupt the fat globule and make it homogeneously smaller in size which in turn allows higher quantities of fat and moisture retention by avoiding syneresis (Lemay and others 1994), higher homogenization pressures might generate a cheese product with less elasticity and firmness (Fox and others 2000), depending on the type of cheese this might be a desirable or non-desirable quality. Escobar and others (2011), showed that applying high pressure homogenization to cheese milk increase the yields of queso fresco, moisture content, and crumbliness which were acceptable for this type of cheese. It has been noticed that homogenization could be detrimental on casein gelation by increasing the number and surface area of fat globules and thus producing a weaker structure (Zamora 2009).

Besides conventional and high pressure homogenization (HPH), there is another type of homogenization that has been applied to milk (Tunick and others 2000). Microfluidization is an efficient high pressure homogenization that has been used for the reduction of particle size especially in pharmaceutical and healthcare industry (McCrae 1994; Fox and others 2000), this technology was patented in 1985 as a high shear homogenization technology for the development of microemulsions processed in a single chamber applying uniform shear forces and thus creating smaller and narrower particle size distributions (Lemay and others 1994; Fox and others 2000; Olson and others 2004; Tobin and others 2015). Microfluidizers generally operate up to 275 MPa, the product enters the system facilitated by a high intensifier pump, divided in two or more micro-streams and directed to a fixed interaction chamber (Figure 2.4) where shear forces, cavitation, and collision are applied with the intention of homogenizing and

decreasing the particle size of the product (Microfluidics 2008). Due to the operating principle, scaling up could be easier (McCrae 1994). Several researchers have studied the effect of microfluidization in cheese milk and consequently in cheese structural properties. Tunick and others (2000), studied how high pressure microfluidization impacts the microstructure of low and full fat Mozzarella cheese processed at different temperatures. They found that at lower temperatures (10 °C) the formation of larger fat globules, in contrast, higher combinations of temperature and pressure, created smaller fat globules. In a similar manner, Van Hekken and others (2007) reported that microfluidization of full fat cheese milk for Mozzarella decreased meltability and flow, a characteristic that is related to the disruption of protein matrix after the application of heat and subsequent melting of fat; this suggest that microfluidization could create not only a smaller particle size of fat but also a less facilitated release since smaller fat globules might be embedded into the protein matrix reducing its ability flow after heating (Diane L. Van Hekken and others 2007).

Microfluidization also was investigated to manufacture Cheddar cheese (Lemay and others 1994), indicating that at higher microfluidization pressures (at equivalent milk fat contents), the reduction in size of the fat globules was more efficient and a weaker curd was obtained, implying there are a greater dispersion of milk fat globules and a less number of caseins available to form the network at the time of curd formation since smaller milk fat globules might be participating in the casein network instead of being entrapped within; furthermore moisture content was higher compared to non-microfluidized controls, reducing the firmness of the final product.

2.4 Cheese texture and rheology

In general, texture is the sensory manifestation of the rheological, structural (geometry and surface) and mechanical properties of food (ISO 1992; Lawless and Heymann 2010). For cheese products, texture is considered one of the principal quality attributes that is intrinsically related to its constituents (casein, fat globules, serum, and minerals) (Gunasekaram and Ak Mehmet 2003), and how they are assembled collectively (Foegeding 2007). Several authors have agreed that cheese texture is highly dependent on its structure at molecular and mesoscale levels, and that ingredients, manufacturing processes and maturation periods might change the structure (Fox and others 2000; Tunick 2000; Lucey and others 2003; Diane L Van Hekken and others 2007; El-Bakry and Sheehan 2014), impacting not only its physicochemical characteristics but also flavor and functionality. Similarly, rheological properties are considered quality attributes of cheese by determining the response or deformation of the product to an applied stress or strain (O'Callaghan and Guinee 2004) and these responses are also considered to be related to the state of their ingredient composition and processing.

The fundamental analysis of texture and rheology in food products is associated with compression and dynamic tests in which samples are deformed under specific conditions to create systematic data for making further inferences about structure, composition, and behavior of the product (Tunick 2000).

Texture Profile Analysis (TPA)

The Texture Profile Analysis or double compression test is a well-known instrumental test to describe and measure the textural characteristic of solid and semi-solid foods (Rosenthal 2010) by mimicking the biting action in the mouth. This test uses the classification proposed by

Szczesniak (1963) in which hardness, cohesiveness, viscosity, and adhesiveness were presented as basic or primary parameters and brittleness, chewiness and gumminess were presented as secondary parameters. Nowadays elasticity has been renamed into springiness and brittleness into fracturability (Texture Technologies), Table 2.2 defines the principal and secondary parameters in Texture Profile Analysis. For the measurement of cheese texture using the TPA analysis, samples of cheese are usually compressed between cylinder flat-ended probes in about 50 – 80% of their original height (Gunasekaram and Ak Mehmet 2003), the displacement of the probe generates a force/distance or time curve, called texture profile, where the primary parameters can be identified (Figure 2.5) Texture Profile Analysis have been used in literature to describe variations in formulation, processing parameters, and to compare between different type of cheeses. For example, Rudan and others (1998) studied the effect of fat particle size by the homogenization of milk and milk standardized with homogenized cream for the preparation of Mozzarella cheese, finding that only cohesiveness increased with the homogenization of both milk and cream compared to non-homogenized samples. Similarly, Tunick and others (1995) compared the textural parameters in Mozzarella cheeses made of homogenized and unhomogenized milk, indicating that higher homogenization pressures increased hardness due to a more crosslinks between casein and fat particles in which fat now might be actively participating in the casein matrix of cheese also decreasing its capacity to stretch.

Moreover, TPA has been used to determine differences in texture due to changes in chemical composition of cheese, specifically changes in fat content (low fat vs reduced fat) (Zheng and others 2016a), proving to be efficient to detect textural differences. Similarly, TPA was used to compare texture parameters in cheeses processed under enzymatic vs acid coagulation conditions (Farkye and others 1995); processed with different levels of calcium

chloride (Ayala-Bribiesca and others 2016) and also was used to compare miniature lab-scale cheeses with commercial brands.

This evidence on TPA analysis supports that ingredient composition and processing parameters, as well as other variables such as coagulation conditions or addition of calcium chloride, have an impact on cheese structure and texture which might be effectively evaluated by TPA analysis.

Rheology - Small Amplitude Oscillatory Shear (SAOS)

Dynamic rheology is also used to describe textural properties of cheese on the short-range interactions (structure of casein particles) (Tunick 2000). The rheological behavior of cheese is viscoelastic, exhibiting both elastic (solid) and viscous (liquid) characteristics at the same time, this behavior is dictated by the ingredient composition and how the structure is linked together to form a network of proteins in which fat particles are entrapped to form clear and visible macrostructure (Subramanian and Gunasekaran 1997; O'Callaghan and Guinee 2004). Small Amplitude Oscillatory Shear (SAOS) analysis is frequently used to determine the rheological characteristics in cheese. This analysis is a subset from what is called Dynamic Mechanical Analysis, which is a technique where small deformation (strain or stress) is applied in a cyclic oscillating or sinusoidal manner. For SAOS test, the deformation is usually between 1 – 5% and this small strain or stress allows to record responsive measurements within the Linear Viscoelastic Region (LVR) (Figure 2.6) in which the stress response (σ) will be proportional to the applied constant strain amplitude (γ_0) (Eq. 1) and frequency-dependent (ω) properties such as elastic (storage) modulus ($G'(\omega)$) or energy stored per cycle; viscous (loss) modulus ($G''(\omega)$) or energy dissipated per cycle; $\tan \delta$ (Eq. 2) that describes the relatively degree of

viscoelasticity; and complex viscosity (η^*), can be recorded (Gunasekaran and Ak 2000; Gunasekaram and Ak Mehmet 2003; Norton and others 2011).

$$\sigma(t) = \gamma_0 G'(\omega) \sin(\omega t) + \gamma_0 G''(\omega) \cos(\omega t) \quad [\text{Eq.1}]$$

$$\tan \delta = G''/G' \quad [\text{Eq.2}]$$

To determine the Linear Viscoelastic Region strain or stress sweeps can be conducted, which is a type SAOS test that records the rheological moduli at a fixed frequency and increments of strain or stress. The Linear Viscoelastic Region serves to identify the strain or stress level at which the responsive measurements will be proportional, in other words, the point at which the shear moduli is not dependent on the input variable (Gunasekaram and Ak Mehmet 2003). Gunasekaram and Ak Mehmet (2003) described the different types of SAOS applied to cheese analysis. Table 2.3 summarizes the main characteristics of the different types of SAOS.

Several studies have analyzed the rheological properties of cheese in order to determine the effect of formulation and processing parameters in texture and structure (Tunick and others 1990; Sanchez and others 1995; Tunick and Van Hekken 2002; Diane L Van Hekken and others 2007; Rogers and others 2009; Rogers and others 2010). Van Hekken and others (2007) studied the rheology of low fat and full fat Mozzarella cheese made from microfluidized milk using strain sweep test to determine the Linear Viscoelastic Region, and frequency sweeps to measure the shear moduli. It was found that different combinations of heat treatment followed by various microfluidization pressures, altered the viscoelastic properties of Mozzarella cheese, finding more differences in storage modulus (G') at lower temperatures and pressures (10°C/34MPa and 10°C/103) compared to samples processed at all temperatures and higher microfluidization pressure (10, 43, 54°C/172 MPa) which were very similar in their rheological behavior,

explaining that at higher homogenization pressures the lipid interaction with the casein micelle is stronger. In another example Sanchez and others (1995) determined the rheological properties of a high fat model acid fresh cheese processed at various levels of homogenization pressures (from 10 to 24 MPa) to evaluate the effect of fat globule size measured by image analysis system; this group also used frequency sweep test to analyze the shear moduli. They observed that G' increased as the fat particle size decreased, creating a firmer structure, explaining that at higher homogenization pressures finer and abundant fat particles were created (called pseudo proteins) allowing more protein-protein interactions and thus more elastic bonds were formed. On the other hand they did not find a clear relationship between milk fat globule size and G'' .

Rheological properties and microstructure of Cheddar cheese have also been reported. Rogers and others (2010) study the effect of different levels of fat content (3 – 33% wt/wt) in Cheddar cheese microstructure. They conducted stress sweeps to determine the Linear Viscoelastic Region, and controlled temperature frequency sweeps (10, 15 20 and 25°C) for measurement analysis, finding changes in the shear moduli at 10°C, but minimal changes at 25°C when comparing cheeses at different fat content. There was a decrease in G' when measured from 10°C to 15°C owing to a phase change in fat from solid to fluid. Also, it was indicated that gel network played an important role in these results since a linear decrease in G' was observed in low fat cheeses (10-15%).

2.5 Vitamin D fortification and bioaccessibility in cheese

General aspects of vitamin D

Vitamin D is a liposoluble vitamin produced from sterols in the body by the action of ultraviolet light on the skin (Combs 2012). It is known principally for participate in calcium and phosphorus metabolism helping to maintain bone health (Ball 1998), and for contributing to chemical processes related to the brain, prostate, breast colon tissues and immune cells, among others. Deficiencies of vitamin D have been related to osteoporosis, muscle weakness, depression of the immune system, the risk of some types of cancer, cardiovascular disease, and other disorders like depression (Ahonen and others 2000; Garland and others 2006; Holick 2006; Holick 2007; Penckofer and others 2010; Urashima and others 2010; Pilz and others 2011). There are two forms of vitamin D identified, cholecalciferol (D₃) and ergocalciferol (D₂) (Figure 2.7) these molecules differ in their chemical structure since ergocalciferol (D₂) have an extra double bond between carbon 22 and 23 and a methyl group on carbon 24 (Holick 2010). Although they are considered equivalent, there are some discrepancies on which one has a higher biological value (Trang and others 1998; Armas and others 2004; Institute of Medicine 2011; Combs 2012).

Vitamin D is naturally found in very few food products; the major source comes from the exposure of skin to sunlight. However, humans can obtain vitamin D from other sources such as dietary supplements and fortified foods. The precursor of vitamin D₂ (ergosterol) is found naturally in some plants, fungi, and yeast, and D₃ (cholecalciferol) is mainly found in animal sources such as fish oils, cod, tuna, cow, pig and chicken livers; eggs and milk (the concentration in the last two depend on animal feed composition) (Holick 2010; Combs 2012).

Since the amount of vitamin D is relatively low from natural dietary sources, food fortification is a common practice not only to target micronutrient deficiency but also to improve the acceptability and marketability of food products (The Dairy Practices Council 2001; Calvo and others 2004). Initially in the United States, fortification of milk with vitamin D started as a program to prevent rickets (bone disease) in children and is optional for milk. Other products such as breakfast cereals and juices fortified with calcium, and milk substitutes beverages contain fortified vitamin D (Calvo and others 2004).

Vitamin D deficiency

According to literature, approximately one billion people around the world have vitamin D deficiency or insufficiency (Holick 2007; Leskauskaitė and Jasutienė 2016). Lips (2010) described the vitamin D status around the different continents, reporting that in European countries the vitamin D deficiency mostly depended on the latitude or the degree of sun exposure and the ethnicity (minorities, especially those non-native to the region). In the Middle Eastern and Asian regions, vitamin D deficiency was correlated with the clothing style and degree of exposure to sunlight. India and Saudi Arabia reported low values of serum 25 hydroxyvitamin D, being the latter with the lowest plasma concentration. In North America and Latin America, the vitamin D deficiency is not very common. However vitamin D insufficiency has been detected in North America, and more studies to gather information need to be conducted in Latin-American regions. In Africa and Oceania, although the data had a high variation, lower values of serum 25 hydroxyvitamin D were related to the latitude, ethnicity, and age. It is important to mention that at a global level, these studies provide a rough idea of the current vitamin D status, but the

assessment and methods of analysis might vary among countries, so an exact comparison could not be shown (Lips 2010).

Cheese fortified with vitamin D

The link of vitamin D deficiency with the development of chronic diseases is increasing awareness of the fact that humans are not ingesting or synthesizing adequate amounts of vitamin D (Tippetts and others 2012). Food fortification with vitamin D is becoming a good option to diversify the currently available vitamin D fortified products (milk, cereals, and beverages) and to reach out to target high-risk populations.

It has been stated that the major source of dietary vitamin D is fortified milk (Banville and others 2000). However, the consumption of milk in the United States have decreased in the past 40 years (Calvo 2000) requiring the availability of diverse sources of vitamin D. Dairy products are considered a good vehicle for micronutrient fortification in terms of quality control, cost, consumption and stability of the added nutrients (Arora and others 2014) and several authors have discussed the benefits of fortifying cheese with vitamin D. Wagner and others (2008) evidenced that cheese is a good vehicle for fortification when comparing the bioavailability of a fortified hard cheese against supplementation by measuring the serum 25-hydroxivitamin D in eighty adults, finding that both treatments were equally bioavailable. Similarly, Johnson and others (2005) studied the bioavailability of vitamin D from fortified process cheese in the elderly, concluding that vitamin D found in process cheese is bioavailable, but the improvement of serum 25-hydroxivitamin D in the elderly might also depend on sunlight exposure. Other studies in vitamin D₃ fortification, mostly in Cheddar cheeses have been

conducted to evaluate the feasibility of fortification (Banville and others 2000; Kazmi and others 2007; Wagner and others 2008; Ganesan and others 2011), considering diverse concentrations of vitamin D₃ ranging from 200 – 400 – 2800 IU of vitamin D₃ per serving of cheese (28 g – 30 g) and diverse forms of vitamin D carrier (i.e. emulsions, liposomes, crystalline form, oil, and premix). Aging time of cheeses varied as well, retaining high percentages of vitamin D₃ ranging from 40 – 97% (up to twelve months), which is a promising result for the feasibility on cheese fortification with vitamin D₃. Likewise, fortification of vitamin D₃ was analyzed in pasteurized process cheese (Upreti and others 2002), in which no loss of vitamin D₃ nor changes in flavor were detected during nine months of storage.

Microstructure, fortification, and bioaccessibility

As previously stated, food structure can play a major role in micronutrients bioaccessibility and bioavailability, and so is another important factor to consider when planning for fortification strategies in the control of micronutrient malnutrition or developing new functional products. Insufficient information is available about the relationship between cheese structure and microstructure with the availability of micronutrients. Recently Ayala-Bribiesca and others (2016) studied how high calcium and very high calcium enrichment in Cheddar cheese affects the matrix structure and subsequent lipid bioaccessibility. It was found that the addition of higher levels of calcium changed the aggregation of milk fat globules making the protein structure more stringy. By higher additions of CaCl₂, moisture content decreased since more whey was expelled after pressing; this caused that protein and fat were more concentrated in the matrix, increasing hardness and compactness of cheese structure, at the same time this

caused more aggregation of the milk fat globules which seem to be bigger under SEM micrographs. During *in-vitro* digestion, lipolysis progressed faster from the high calcium samples compared to the control. However, at the end of the digestion period, the very high calcium cheese had the lowest mass disintegration index, suggesting that the rate of lipolysis depends on calcium and the cheese matrix as well. Besides this result on cheese microstructure and bioaccessibility, there is a lack of evidence regarding how cheese microstructure would affect the vitamin D bioaccessibility in a fortified cheese product. Therefore there is a critical need to study the relationship between the structure of cheese and bioaccessibility of micronutrients.

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2.7 Tables and Figures

Table 2.1 Recent studies relating food matrix, processing or formulation parameters and nutrient availability.

Nutrient	Food matrix	Processing / formulation	Method for assessing digestibility	Findings	Reference
Iron	Filamentous whey protein hydrogels and particulate whey protein hydrogels	β -lactoglobulin gels with 1 M FeSO_4 solution addition. Cold-induced gelation. Filamentous vs Particulate gels created dependent on iron/protein ratios.	Bioaccessibility by two stage <i>in vitro</i> digestion model. Gastric and small intestine. Followed by a simulated intracellular absorption on the intestine wall using Caco-2 cell from colorectal carcinoma.	Dependence on gel microstructure on iron release at different pH (gastric and intestine). In gastric conditions release from filamentous gels was lower than particulate. During intestinal conditions both gels had a rapid initial iron release however filamentous gels had a smaller sustained additional release compared to particulate gels.	(Remondet to and others 2004)
β -carotene	Raw, gently cooked and intensely cooked carrots.	Thermal processing and mechanical breakdown to generate different particle sizes.	Bioaccessibility by two stage <i>in vitro</i> digestion model. Gastric and small intestine.	Thermal processes affected the carrot tissue particle size and this to the overall bioaccessibility of β -carotene,. Bioaccessibility seemed to be higher at smaller particle size and high thermal processing.	(Lemmens and others 2010)
β -carotene	Carrot tissue, <125 μm and between 500 and 4000 μm (particle size)	Heat treatment and time from 90 to 120 $^{\circ}\text{C}$ from 0 to 60 min.	Bioaccessibility by two stage <i>in vitro</i> digestion model. Gastric and small intestine.	Bioaccessibility of β -carotene increased at higher temperatures and smaller particle size.	(Lemmens and others 2011)

Table 2.1 (cont.)

Carotenoid species	Tomato pulps made by red, orange and yellow ripe tomatoes	High pressure homogenization (20, 50 and 100 MPa)	Bioaccessibility by two stage <i>in vitro</i> digestion model. Gastric and small intestine.	Consistency of all tomato varieties increased at higher homogenization pressures and carotenoids bioaccessibility decrease due to the strengthening of the fiber network entrapping the compounds.	(Panozzo and others 2013)
Lipids	Crushed almonds Almond milk Almond cream Almond oil NaCas – almond oil Span 80 – almond oil Tween 60 – almond oil	Crushing and shear.	Bioavailability by <i>in vivo</i> studies using female rats	Crushed almonds and emulsified almond lipids had a different digestibility. It was shown that lipids from crushed almonds remained either trapped or emulsified in the chime. Meanwhile, almond oil coming from emulsions were emulsified in the stomach. All samples had diverse behavior during gastric digestion which in turn impacted apparent ileal fatty acid digestibility.	(Gallier and others 2014)
Vitamin D ₃	Oil-in-water nanoemulsions. Oil phase: corn oil, fish oil, mineral oil or orange oil. Aqueous phase: 2% surfactant with buffer solution.	High Pressure Homogenization 12,000 PSI and type of oil phase carrier.	Bioaccessibility by three stage <i>in vitro</i> digestion. Mouth, gastric and small intestine.	Higher bioaccessibility of vitamin D ₃ form low chain triglycerides (corn, oil and fish oil) than those with medium chain triglycerides (orange and mineral oil)	(Ozturk and others 2015)

Table 2.1 (cont)

Lipids	Instant noodles fried in palm oil stabilized with different emulsifiers.	Frying at 145 °C for 100 seconds.	Three stage <i>in vitro</i> digestion. Mouth, gastric and small intestine to see changes in lipid digestion patterns.	Lipid particle size prepared with yolk lecithin were smaller than with other emulsifiers at the end of <i>in vitro</i> digestion and lipid emulsified with Tween 20 and caseinate were about 1-2 times larger than the others. Fatty acid composition and free fatty acid content were similar between samples.	(Hur and others 2015)
Vitamin C and phenolic compounds.	Fruit juice-based beverages Fruit mixture with whole milk soy milk.	High-intensity pulsed electric fields High pressure processing Thermal treatment (TT) 90°C for 60 s.	Bioaccessibility by two stage <i>in vitro</i> digestion model. Gastric and small intestine with dialysis.	Vitamin C bioaccessibility decreased by thermal processes with both whole milk and soy milk For some groups of phenolic compounds, the bioaccessibility increased or decreased according to processing and food matrix.	(Rodríguez-Roque and others 2015)
Lipids	Cheddar type cheese	Cheddar type cheese with addition of calcium (4 or 12 g kg ⁻¹ curd).	Bioaccessibility by three stage <i>in vitro</i> digestion. Mouth, gastric and small intestine.	Microstructure of cheese was modified by high and very high levels of calcium specially the aggregation of milk fat globules. The lowest lipolysis rate at the end of intestinal phase was from the very high calcium samples suggesting the rate of lipolysis depends on calcium and the cheese matrix.	(Ayala-Bribiesca and others 2016)

Table 2.2 Primary and secondary parameters in Texture Profile Analysis.

TPA primary parameters	Definition	Measurement
Hardness (N)	Is the maximum force identified at the first compression to attain a given deformation.	P1
Cohesiveness (-)	Strength of the internal bonds making up the body of the product.	A2/A1
Springiness (%)	Ability of the product to return to its original height after the deforming force is removed.	Dis 2 / Dis 1 or Time 2 / Time 1
Adhesiveness (J) ¹	Work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes in contact.	A3
TPA secondary parameters	Definition	Measurement
Fracturability (N)	Force at which the material fractures, is determined as the significant break on the first bite. Is related to hardness and cohesiveness.	F1
Gumminess (N)	Energy required to disintegrate a semisolid food product. Related also to hardness and cohesiveness.	Hardness Cohesiveness
Chewiness (J)	Energy required to chew a solid food until ready for swallowing.	Hardness Cohesiveness * Springiness

Source: Szczesniak (1963), Gunasekaram and Ak Mehment (2003)

¹Adhesiveness might require another type of analysis (depending on the type of matrix) instead of using double compression test (Texture Technologies).

Table 2.3 Main characteristics of the Small Amplitude Oscillatory Shear tests.

Type of SAOS	Description
Strain or Stress Sweep	<ul style="list-style-type: none">▪ Increasing strain or stress at a constant frequency.▪ 1-5% deformation applied.▪ Regularly used to determine LVR.
Frequency Sweep	<ul style="list-style-type: none">▪ Fixed amplitude sinusoidal strain (or stress).▪ Strain must be within the LVR.▪ Frequency range is used.▪ Useful to characterize viscoelastic behavior.
Temperature Sweep	<ul style="list-style-type: none">▪ Constant strain (or stress).▪ Constant frequency.▪ Temperature range is used.▪ Regularly used to investigate phase transitions.
Time Sweep	<ul style="list-style-type: none">▪ Constant strain (or stress) amplitude.▪ Constant frequency.▪ Combined effects of time and temperature, e.g. Milk gelation time.

Source: Gunasekaram and Ak Mehment (2003)

Figure 2.1 Structural dimension and approximate size of some food components

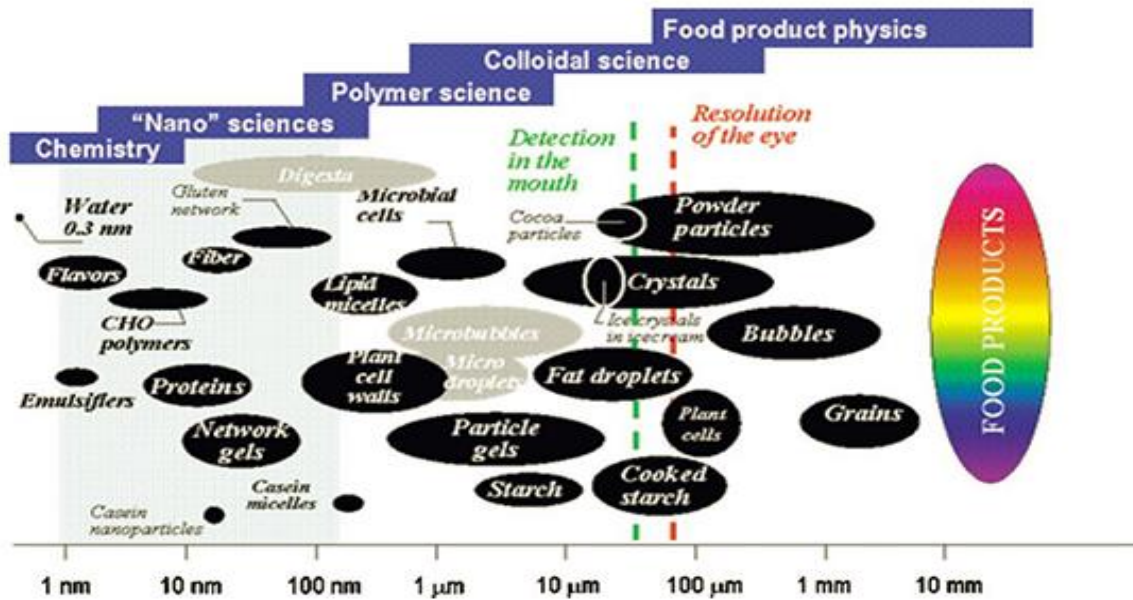


Illustration of the structural dimensions of some food components that interact to form part of food matrices (Aguilera 2012).

Figure 2.2 Scheme of cheese microstructure.

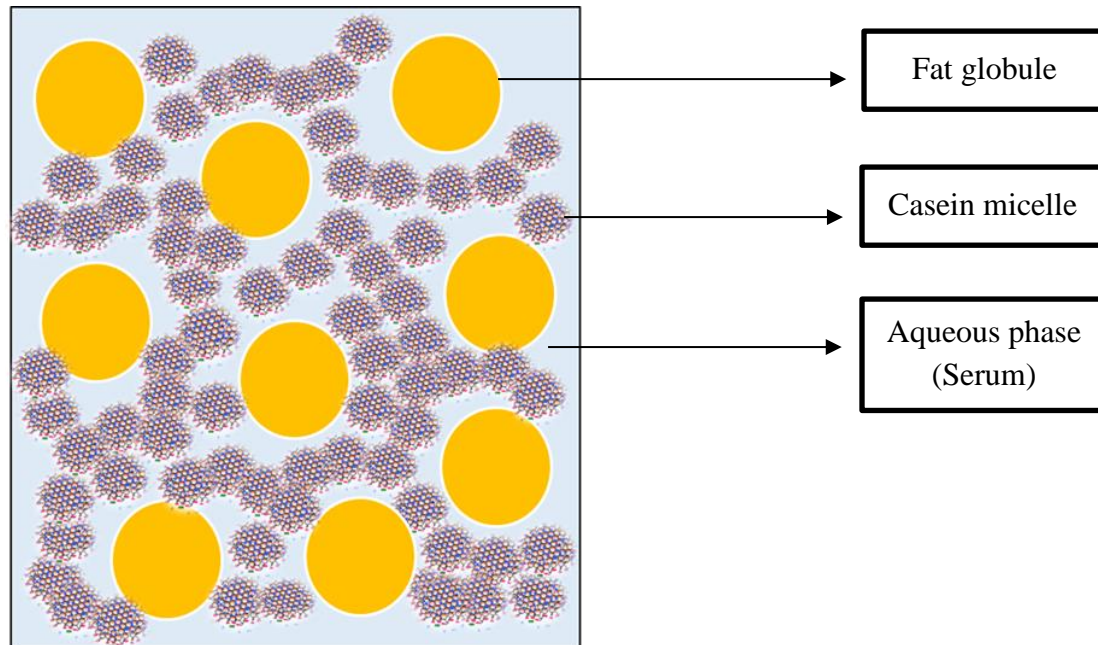


Illustration of the cheese microstructure in which casein micelles forms a protein network in which fat globules and serum are entrapped

Figure 2.3. Casein micelle visualized under cryo-transmission electron microscopy

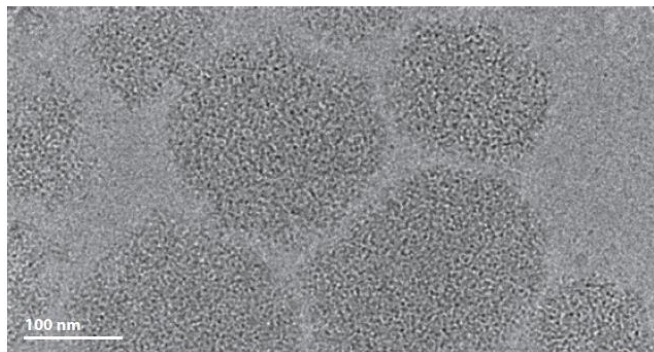
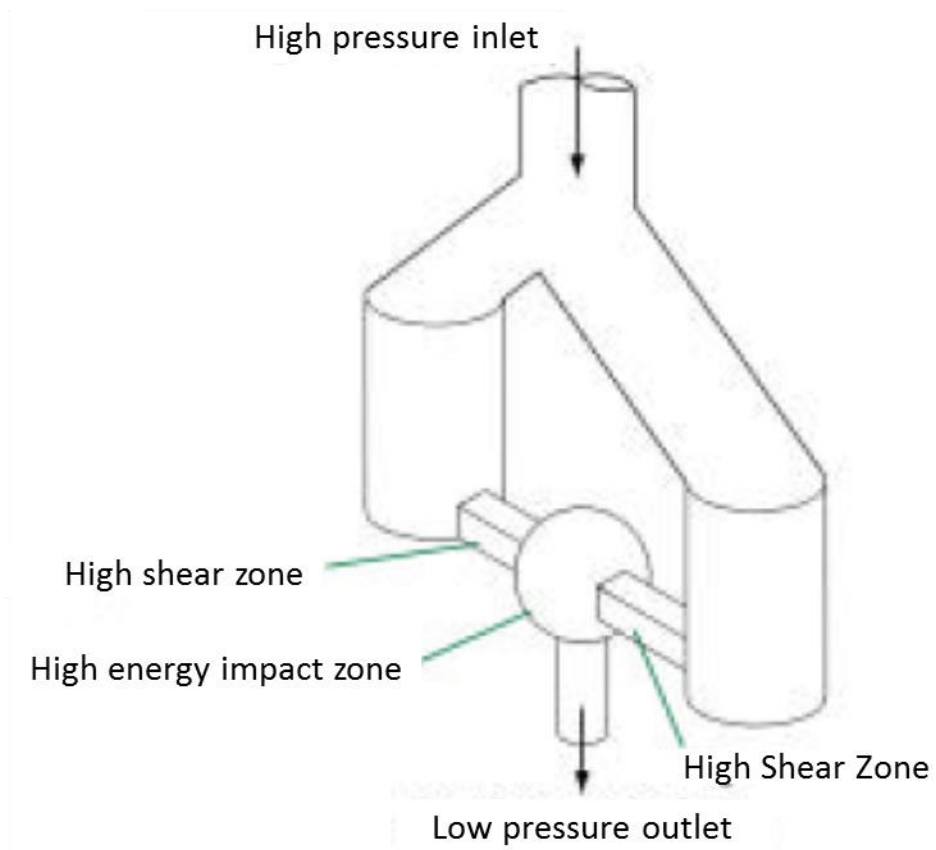


Illustration of casein micelles under microscope (Dalglish and Corredig 2012).

Figure 2.4 Representation of the single slotted interaction chamber used in microfluidization



Source: Microfluidics (2008)

Figure 2.5 Texture profile of the double compression test.

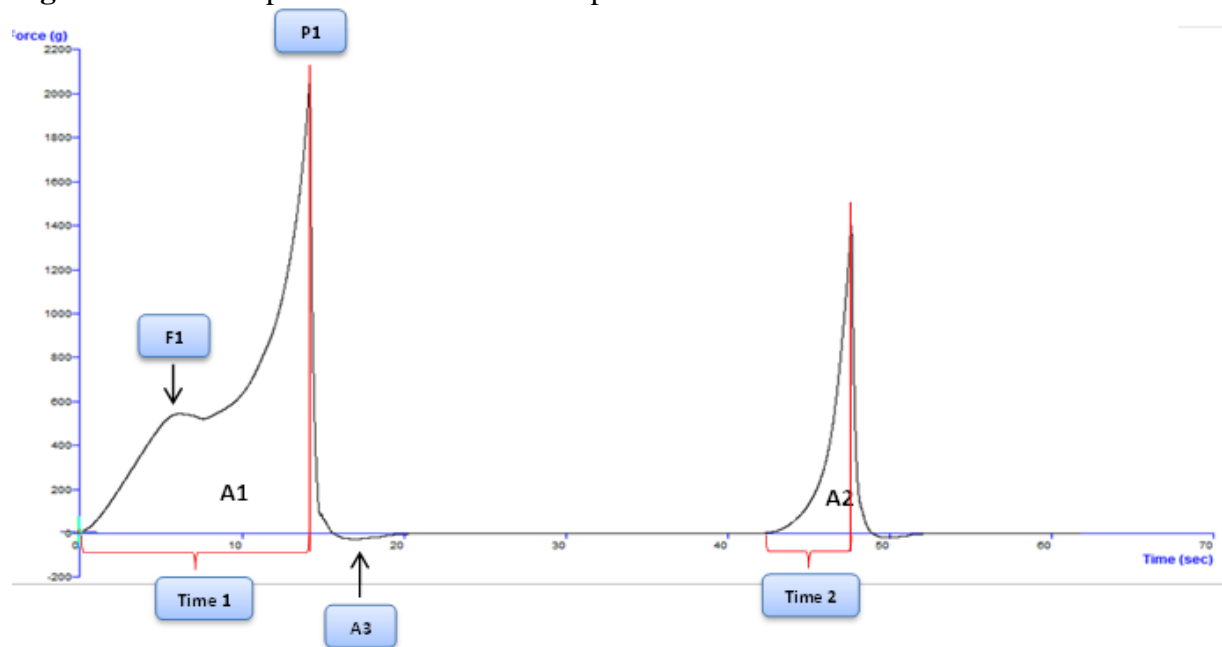
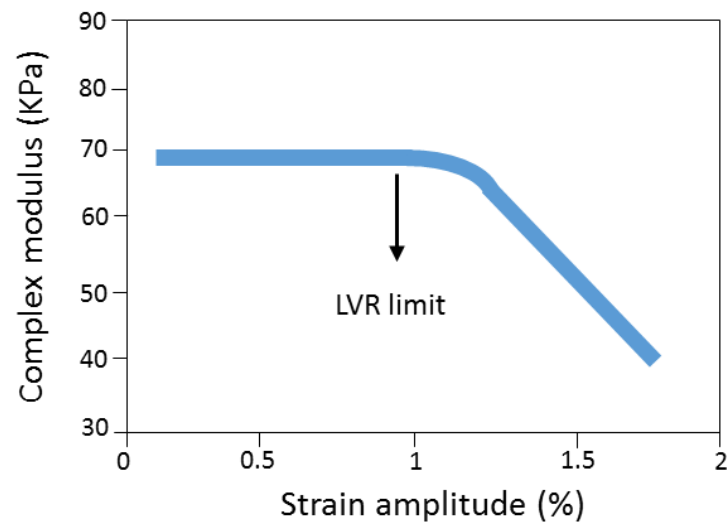


Illustration of the Texture Profile Analysis performed in a model fresh cheese, plotting Force (g) against Time (sec). P1 corresponds to Hardness, F1 to Fracturability, A1 and A2 indicates area under the curve 1 and 2 respectively, A3 is referred as adhesiveness.

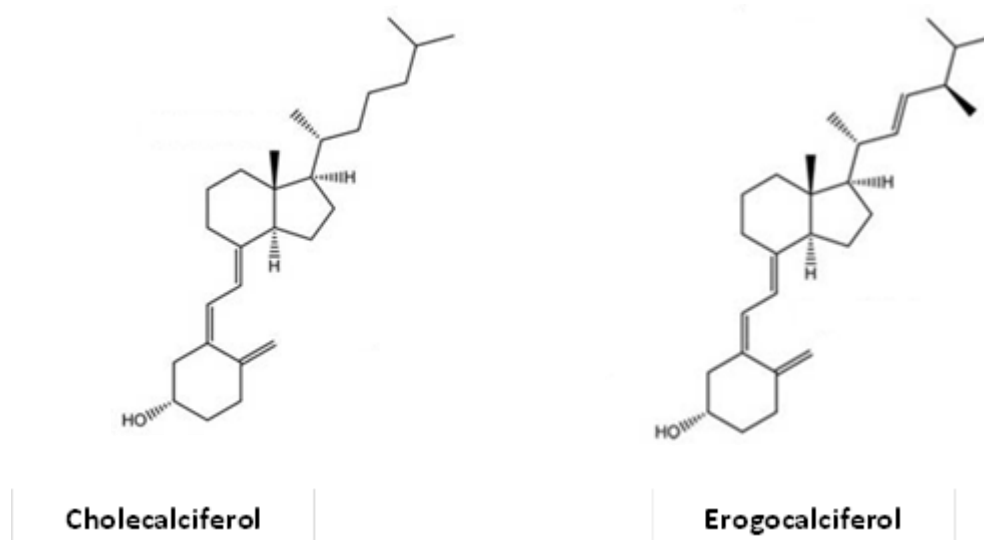
Adapted from Szczesniak (1963), Gunasekaram and Ak Mehment (2003)

Figure 2.6 Representation of the Linear Viscoelastic Region by strain sweep test.



Source: Gunasekaram and Ak Mehment (2003)

Figure 2.7 Cholecalciferol and ergocalciferol chemical structure.



Ergocalciferol has one extra double bond between carbon 22 and 23 and a methyl group in carbon 24

Chapter 3. Physical properties and microstructure of a model fresh cheese

3.1 Abstract

A model fresh cheese was used as an example of soft solid matrix to evaluate the structure and microstructure created by variations in formulation (protein to fat ratios – P/F) and processing parameters (homogenization pressures using microfluidization). It was hypothesized that increasing P/F ratios (0.9, 1.3, 1.7 and 2) and homogenization pressures (17, 50, 75 and 150 MPa) the particle size of the cheese milk emulsion will decrease, resulting in a more rigid and elastic matrix with a smaller pore size. Samples were prepared by skim milk powder (SMP) and anhydrous milk fat (AMF) at the appropriate ratios and reconstituted with DI water followed by homogenization and curd formation by induced-heat acidification. Particle size of the cheese milk emulsion was analyzed by laser diffraction. Texture and rheological properties were analyzed using large and small deformations by Texture Profile Analysis (TPA) and Small Amplitude Oscillatory Shear (SAOS) respectively. Pore size and porosity were assessed by analyzing images obtained by Environmental Scanning Electron Microscopy (ESEM). Results show that smaller particles sizes were created in the cheese milk emulsion as P/F and homogenization pressures increased. Similarly, TPA parameters increased as these two factors increased; however, no clear trend was determined for small deformations. Although differences in particle size were seen, the porosity and pore size did not show significant differences between samples. Overall protein to fat ratio had a more prominent effect on structure and properties of the model cheese than homogenization pressure, suggesting that the re-arrangement of protein micelles into a continuously aggregated matrix affected by heat-induced coagulation is critical for the structure development. Understanding the matrix conformation in complex food systems

such as soft solid is important for the engineering of food products with specific functional characteristics.

3.2 Introduction

There is an increased interest in the study of food structure from a functional and nutritive standpoint as a new window for innovation. This idea comes from the fact that processed foods are complex matrices with components interacting below 100 μm (Aguilera 2005; Parada and Aguilera 2007; Sensoy 2014a), and that composition as well as processing parameters are responsible for building up these interactions which will define the final product characteristics such as texture, flavor, appearance, nutritional composition, functionality and also absorption during digestion (Aguilera and Stanley 1999; Lamothe and others 2012) . The complexity of a processed food product is given by the variety of phases that can be found within a single matrix, for example, many commercially available food products show a combination of solid and liquid-like properties (Stokes 2012) classified as soft solids (Aguilera and Stanley 1999; Stokes and Frith 2008) (i.e. jellies, jams, processed meat and dairy products). Given their versatility of developing structures, soft solids have been extensively investigated from a technological perspective. However few studies have related the structural parameters of a complex food system with nutrient availability (Remondetto and others 2004; Zheng and others 2016b).

Dairy products, specifically cheese is considered a complex nutrient-dense system capable of creating diverse structures due to changes in their composition (i.e. protein, fat, moisture and minerals) and processing parameters (i.e. homogenization, acidification, enzymatic action, pasteurization, filtration) (Kielczewska and Kruk 2006; Everett 2007; Rogers and others

2009; Hickey and others 2015), these factors are key to determine cheese quality and identity. Most of the studies found in literature are related to the structural and rheological evaluation of hard and semi-hard cheeses (Tunick and others 1990; Ma and others 1997; Rogers and others 2009; Rogers and others 2010), few of them describe the structural properties of fresh cheese especially made by acid coagulation. Sanchez and others (200) studied the fat globule size and rheological properties of acid fresh cheeses homogenized up to 24 MPa. Other studies have analyzed the structures of fresh cheese comparing raw vs pasteurized milk or the addition of diverse levels of sodium caseinate (Lobato-Calleros and others 2000; Diane L Van Hekken and others 2007); however there is no information about the structure formation of heat-induced acid coagulated cheese processed at various protein to fat ratios and homogenization pressures by microfluidization.

The objective of this study is to evaluate the structure and microstructure of a model fresh cheese processed a various protein to fat ratios and homogenization pressures using microfluidization. Particle size, Textural Profile Analysis (TPA), Small Amplitude Oscillatory Shear (SAOS) rheology and Environmental Scanning Electron Microscopy (ESEM) analysis were used to evaluate the structural and microstructural characteristics of the matrix, which will be used for further studies on bioaccessibility.

3.3 Materials and Methods

Soft-solid matrix (model fresh cheese) preparation

Model fresh cheese was prepared in a similar manner as “Queso Blanco” (Kosikowski 1977; Fox and others 2000) with some modifications (Figure 3.1). Four different formulations were analyzed, varying the protein to fat ratio (0.9, 1.3, 1.7 and 2) (Fox and McSweeney 2004;

Guinee and others 2007; Phan and others 2008) and microfluidization pressure of milk emulsion (17, 50, 75 and 150 MPa) (Parnell-Clunies and others 1985; McCrae 1994; Fox and others 2000; Olson and others 2004); these levels were selected based on previous studies found in literature related to the effect of protein to fat ratio in cheese and cheese milk parameters, as well as preliminary analysis on the appropriate ratios that worked well with the microfluidizer. All formulations were prepared to maintain the same amount of solid content. Table 3.1 and 3.2 show formulation based on different protein to fat ratios and experimental designed respectively.

The designated amount of skim milk powder (SMP, MP Biomedicals, LLC., Solon, OH., U.S.A.) was reconstituted in 1300 mL of deionized (DI) water at 60°C (Ann Augustin and Clarke 2011) by mixing them in a professional mixer (KitchenAid® Professional 5Q Mixer, KitchenAid, Benton Harbor, MI., U.S.A) at low to medium shear (level 2). SMP was added progressively to ensure complete dispersibility and mixed for 5 minutes. Anhydrous milk fat (AMF, Danish Maid Butter Co., Chicago IL., U.S.A.) was pre-warmed in a water bath at 60°C and added gradually to the reconstituted milk in a pre-homogenization step, using an IKA T-25 Digital High-Speed Homogenizer (IKA Works Inc., Wilmington, N.C., U.S.A.) at 8000 rpm. Once added, rotation speed was brought up to 11000 rpm for additional mixing for 5 minutes. Emulsion was degassed under 170 mm Hg vacuum for 10 minutes to remove foam, and microfluidized using a M-110P microfluidizer (MircfluidicsTM, Weswood, MA., U.S.A.) equipped with a Y-chamber. After microfluidization two samples of 20 mL were obtained for particle size analysis and the rest of the emulsion was kept under refrigeration at 4°C for approximately 6 hours until coagulation step and subsequent cheese preparation. All cheese samples were made the same day as the emulsion preparation. Cheese milk emulsion was heated in a water bath until reaching 82°C (Siapantas and Kosikowski 1967; Kosikowski 1977; Torres

and Chandan 1981; Fox and McSweeney 2004). 0.02% w/w of calcium chloride (Fisher Scientific Company LLC., Hanover Park, IL.) was added to the cheese milk emulsion as stated in Cheese and Related Cheese Products, Title 21 *Code of Federal Regulations*, Pt. 133.89., (2016) to compensate calcium losses during acid coagulation (Tunick 1987). About 50 mL of a 1:10 solution of glacial acetic acid 99% USP/FCC donated by AFCO (AFCO., Chambersburg, PA., U.S.A) was added to the heated milk to reach a pH between 5.0 and 5.2 (Farkye 2004; Phadungath 2005) forming the cheese curd instantly. Part of the whey (400 mL), was drained and 2.5 %,w/w of sodium chloride (crystalline/certified ACS,Fisher Scientific., Fair Lawn, NJ, U.S.A) was added (Kosikowski 1977; Kindstedt 2005; Tunick and others 2012). Cheese curd was drained and pressed overnight (15 h) using an unbleached 100% cotton cheese cloth grade 50 (Bassiloff Co., New York, NY., U.S.A) and a Deluxe Dutch Cheese Press (The Sausage Maker Inc., Buffalo, NY., U.S.A). Pressing was performed with a 4.5 Kg weight (~3.5 KPa). After pressing, samples were stored at 4°C in aluminum foil and sealed plastic bags to avoid moisture loss. Samples were prepared in triplicate. Moisture content measurement, Texture Profile Analysis (TPA) and Small Amplitude Oscillatory Shear (SAOS) were conducted on the next day of sample preparation to characterize the physical and rheological properties of the model cheeses. Environmental Scanning Electron Microscopy (ESEM) and Confocal Laser Scanning Microscopy (CLSM) were performed within 2-3 days after sample preparation to analyze pore size, porosity, and distribution of fat globules.

Cheese milk emulsion particle size

Cheese milk emulsion particle size (d_{43}) was measured using a Shimadzu SALD-2300 Laser Diffraction Particle Size Analyzer equipped with a SALD-MS23 Sampler (Shimadzu

Scientific Instruments, Inc., Columbia, MD). Measurements were conducted at room temperature following the microfluidization. Drops of the liquid emulsions were dispensed into sampler water bath with circulating deionized water until acceptable light absorbance was reached. Measurement was taken in duplicate for each replicate using a refractive index of 1.45 ± 0.02 (Olson and others 2004).

Moisture content

Moisture content was measured by using a Mettler Toledo Moisture Analyzer HB43-S (Mettler Toledo International Inc., Columbus, OH., U.S.A). .5 – 2.6 g of grated model cheese was placed in an aluminum sample pan for moisture analyzer (Mettler Toledo International Inc.) and a pre-set method for cheese was chosen from the equipment database. Measurements were taken in duplicate for each replicate.

Texture Profile Analysis

Texture Profile Analysis (TPA) or double compression test was performed to evaluate the textural properties of model cheese samples (Gunasekaram and Ak Mehmet 2003). Model cheese samples were cut using a sharp carbon blade into 2x2x2 cm cubes perpendicular to the direction in which sample was pressed (Chen and others 1979). A digital caliper was used to measure dimensions of each cube. Four samples were taken from each replicate. Test was performed at room temperature (25°C) equilibrating the samples prior analysis for two hours in sealed plastic containers to avoid moisture loss. TA.XT. Plus Texture Analyzer (Stable Micro Systems LTD., Vienna Court, U.K.) was used to conduct the test, TPA settings include 5 Kg loading cell attached to 50 mm diameter flat compression plate; 1 mm/s for test speed, 75% strain and 5

seconds delay between first and second compression (Tunick, Michael H.; Mackey, Kevin L.; Smith, Phillip W.; Holsinger 1991; Tunick and Van Hekken 2002; Diane L Van Hekken and others 2007; Gutiérrez-Méndez and others 2013). Force versus time curves were obtained. Compression force, crosshead travel and peak area data were identified and transformed into hardness, cohesiveness, springiness and fracturability by using the Exponent software (Stable Micro Systems LTD.) (Tunick, Michael H.; Mackey, Kevin L.; Smith, Phillip W.; Holsinger 1991).

Rheology Analysis: Small Amplitude Oscillatory Shear

Small Amplitude Oscillatory Shear test was performed to the model cheese samples, as a nondestructive test that can provide information about structure development under different formulation and processes (Gunasekaram and Ak Mehmet 2003). Samples were cut at 4°C (perpendicular to pressing direction) with a cheese slicer to a thickness 4 mm. They were cut into a round shape with a 30 mm diameter plain edge round cutter. Three samples were taken from each replicate. The test was performed at room temperature (25°C) equilibrating the samples for one hour in sealed plastic containers to avoid moisture loss prior measurement. An ARES-G2 oscillatory rheometer equipped with an Advanced Peltier System was used for rheological measurements, and TRIOS[®] software was used to collect and analyze data (TA Instruments, New Castle, DE). Linear Viscoelastic Region (LVR) was determined by dynamic strain sweep and viscoelastic behavior of the model cheese samples was determined by frequency sweep, both of them using a stainless steel serrated parallel top plate (25 mm diameter) with a gap between 3.5 – 4 mm (Tunick and others 1990; Gunasekaram and Ak Mehmet 2003; Rogers and others 2010). The axial force was kept constant at the time of measurement. For the dynamic strain

sweep, a constant frequency was used at 10 Hz, with a strain range between 0.1 to 1%, and frequency sweep test was conducted from 0.1 to 10 Hz (Rogers and others 2010) at 0.5% strain based within the LVR. Elastic and viscous modulus (G' and G''), complex viscosity (η) and $\tan \delta$ (ratio of viscous to elastic modulus) were the rheological parameters calculated by the instrument software.

Microstructure analysis

Environmental Scanning Electron Microscopy (ESEM) model Phillips XL30 ESEM-FEG (FEI Company, Waltham, MA., U.S.A) was used to analyze porosity and pore size of the model cheese samples. The method for sample preparation was adapted from Kuo and Lee (2014) with some modifications. 3x2x10 mm sample pieces were cut with a razor blade (perpendicular to sample pressing) one hour before analysis and kept at 4°C in sealed containers. Then, the sample was frozen in liquid nitrogen for about 3-4 minutes, fractured and mounted on the stage. Imaging analysis was taken at 1torr wet mode with a Peltier stage, observed with an accelerated voltage of 20kV and 2000x magnification. Porosity and pore size was analyzed using Matlab (version 7.0.4.356 R14, The Mathworks Inc., Natick, Mass., U.S.A.) and the Matlab Processing Toolbox (Math Works 2014) by using the grey scale thresholding method which converts gray-scale images micrographs into binary-scale images determining the threshold grey tone that resulted in the maximum number of pores that could be identified (Gueven and Hicsasmaz 2013) and thus separating the pore from the matrix. A second threshold was also applied to exclude the protruding area of the sample in the image, by multiplying 1.25 to the grayscale intensity of the first threshold (Kuo and Lee 2014). Porosity and pore size were calculated in volume, so the square root was taken from each result and raised to the power of three to get the data in volume.

Confocal Laser Scanning Microscopy (Zeiss LSM 700 Confocal. Carl Zeiss, Oberkochen, Germany) and imaging analysis ZEN lite software (Carl Zeiss) were used to analyze fat and protein distribution within the sample matrix. The method for sample preparation was taken from Everett and Auty (2008). Samples were cut in 10x10x5 mm at 4 °C with a razor blade (perpendicular to sample pressing) and kept at refrigeration temperature for 1 hour before analysis in tight containers to prevent from drying and fat melting. A fluorescence mixture probe was prepared by mixing 0.02 g/L of nile red (488 nm excitation wavelength) and 0.02 g/L of nile blue (633 nm excitation wavelength) in a mixture of PEG (Sigma-Aldrich, INC., Milwaukee, WI., U.S.A) plus 20µL of distilled water/g. Fifty microliters (50 µL) of the probe mixture were dropped on the sample surface with a resting time of 10 minutes at 4 °C, micrographs were taken at 63 x objective with numerical aperture of 1.4, a confocal pinhole diameter of 0.2 µm and 1.0 µm axial resolution. Fat particles were labeled with nile red and protein was labeled with nile blue.

Statistical analysis

Statistical analysis was performed using Statistical Analysis System software (SAS[®] 2016, SAS Institute Inc., Cari, NC., U.S.A). Analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) were used to analyze statistical differences between all combinations of formulation and homogenization pressure treatments, and for analyzing results in pore size and porosity; all of them with a type I error significance level (α) of 0.05.

3.4 Results and Discussion

Particle size of the cheese milk emulsion

Figures 3.2 and 3.3 show the particle size average diameter d_{43} of the cheese milk emulsion after microfluidization. Variations in protein to fat ratio (P/F) and homogenization pressures had significant effect on the particle size of the cheese milk emulsion ($p < 0.05$). As P/F increased the particle size decreased significantly, this effect was also related to a higher homogenization pressures which resulted in smaller particle size diameters around $0.66 \pm 0.05 \mu\text{m}$. Samples processed at higher P/F ratio and homogenization pressure caused a smaller fat droplet size suggesting a rapid adsorption of the milk protein to the surface of the fat globules, and thus, the steric stabilization of casein evades the droplets to coalescence -due to an abundant protein layer (Dickinson 1997; Lee and others 2009). On the other hand, when the protein to fat ratio and homogenization pressure is smaller, it resulted in bigger particles ($\sim 4.37 \pm 1.55 \mu\text{m}$) compared to high protein to fat ratios and homogenization pressures. As fat content increases, it is expected a higher degree of aggregation between fat particles therefore emulsion particle size tend to be bigger, this result is explained by Tomas and Paquet (1994) who studied how fat to protein ratio affect the droplet size in dairy emulsions, finding that a higher fat contents some aggregation is likely to occur since the relative amount of protein present might not be sufficient to stabilize all fat droplets during homogenization.

There was also a significant interaction ($p < 0.05$) between P/F ratios and homogenization pressure. For the effect on the particle size, P/F ratio displayed clearer trend (Figure 3.3) than homogenization pressure, especially because the samples processed at 50 MPa formed larger particles than the sample processed at 17 MPa.

Texture Profile Analysis in model fresh cheese samples

Protein to fat ratio had a more prominent effect on the dependent variables hardness, cohesiveness, springiness and fracturability than homogenization pressure (Figures 3.4 – 3.7). Hardness was significantly higher for those samples with higher protein to fat ratio (1.7 and 2) which is explained by an increase of the volume fraction of the protein matrix compared to the fat and thus a sample with firmer texture was formed (Fox 1999). Similarly, cohesiveness, springiness and fracturability increased at a higher protein to fat ratios (1.7 and 2), which indicate more strength in the internal bonds due to higher levels of protein so the sample tend to hold together to a greater extent (Tunick and Van Hekken 2002; Kiziloz and others 2009). Conversely for those samples at lower protein to fat ratio (0.9 and 1.3) the higher fat content has been shown to work as filler as well as a lubricant in cheese matrix and higher fat contents may reduce the stress to break the sample and thus result in smaller texture parameters (Fox and others 2000; Dimitreli and Thomareis 2007). This effect has been evident for Mozzarella cheese processed at high homogenization pressures, which shown significantly ($p < 0.05$) lower values in textural parameters (except cohesiveness) attributed to higher fat retention (Jana, A.H; Upadhyay 1991).

The texture analysis was based only on the interaction of proteins and fat in the food matrix. Moisture content was not included since it did not show significant differences among the samples mostly because of the standardized quantity of solids (Everard and others 2011).

On the other hand homogenization, pressure did not show a significant effect on the parameters obtained in TPA analysis. Although no statistical differences were found, there were visible differences between samples at lowest and highest homogenization pressures during curd

formation. Similar to other studies (Dalglish and others 1996), high homogenization pressures causes a weaker formation of the curd, and more shattering was perceived. Higher dispersion of the fat globules in the cheese milk emulsion may have reduced the amount of free casein particles that participate in the formation of a stronger protein network during curd formation (Jana, A H; Upadhyay 1992). Also, it was reported that curd formation by microfluidized cheese milk was brittle with an increase of fines (small and soft casein particles) collected in the whey during Cheddar cheese processing (Lemay and others 1994).

The visible differences in samples processed at various homogenization pressures during curd formation, yet no significant differences in the measured textural properties indicates that there is a need for further evaluation of the processing methods used in this study.

In this study, heat-induced acid coagulation was used, which includes applying a heat treatment at 82°C before the addition of 1:10 glacial acetic acid until pH of 5.1 – 5.3 and curd formation. For heat-induced acid coagulated milk gels, the application of heat causes denaturing of the whey proteins which start interacting with casein micelles. It has been identified that some β -lactoglobulin is associated with k-casein by intermolecular disulfide bonds (Lucey and Singh 1997; Vasbinder and others 2001a), this interaction causes aggregation of caseins at higher pH since the isoelectric point of β -lactoglobulin is ~5.3 (Lucey and others 1988). When an organic acid is added, solubilization of colloidal calcium phosphate within the casein micelles occurs, and there is a re-association of the casein proteins forming continuous protein aggregates rather than aggregated sub-micelles (Heertje and others 1985; Vasbinder and others 2001b). This new conformation of the casein micelles due to heat-induced acidification may minimize the effect of homogenization on textural properties. Thus, significant differences due to homogenization

pressure could not be seen after curd formation and pressing. Therefore, the ratio of protein to fat had a dominant effect on the textural parameters of the model cheese samples.

Small Amplitude Oscillatory Shear

Rheological moduli (G' , G'' , $\tan \delta$) and complex viscosity (η) obtained by frequency sweep test showed some variations among formulations and homogenization pressures. Overall, storage modulus (G') was higher compared to loss modulus (G'') for all samples, and both G' , G'' showed positive dependences with frequency, which might be caused by the formation of an inhomogeneous particulate structure typically found in acid milk gels (Langton and Hermansson 1992; Ozer and others 1998). However, no clear trend was seen between samples in terms of the effects of P/F ratio or homogenization pressure on G' , G'' (Figure 3.8 and 3.9). Similar results were reported by Cobos and others (1995), where no significant differences were found in the rheological properties of acidified milk gels processed in microfluidizer or valve homogenizer concluding that the effect of heat treatment and solid levels were more prominent than homogenization process. Interestingly, the samples at a higher protein to fat ratios (2 P/F) showed G' and G'' spectra to be less variable regardless of homogenization pressure, which means more uniformity in terms of structure. No significant effect was detected for complex viscosity (η) for formulation or homogenization pressure (Figure 3.10).

On the other hand, $\tan \delta$, which is the ratio of G'' over G' , plotted as a function of frequency (ω), ranged from 0.21 to 0.28 and showed that as P/F ratio increased the $\tan \delta$ also increased (Figure 3.11 and 3.12). This indicated that over the applied frequency and strain,

lowest P/F ratio (high fat added) showed less relative viscous properties compared to samples with higher P/F ratios (less fat added).

To analyze statistical differences between rheological parameters, measurements taken at frequencies of 1 Hz (from a range of 1 – 10 Hz) were selected. The results showed that only the effect of the formulation (P/F ratio) was significant ($p < 0.05$) for loss modulus (G'') and $\tan \delta$ (Figures 3.13 and 3.14), and no significant differences were detected for storage modulus (G') and complex viscosity (η). The homogenization pressure nor the interaction between all rheological parameters were significant. For loss modulus (G''), no clear trend was seen, however, samples processed at 1.7 P/F showed to have overall higher loss modulus compared to the other samples.

As previously mentioned, all model fresh cheese samples showed a viscoelastic behavior, with more elastic than viscous characteristics since $\tan \delta$ values were less than 1 (between 0.21 – 0.28). By analyzing samples at 1 Hz, $\tan \delta$ significantly increased ($p < 0.05$) at 1.7 and 2 P/F (Figure 3.13); indicating that at lowest P/F ratios (high fat added) less relative viscous properties were seen, and overall formed a weaker structure. These results might have been overshadowed by the coagulation process and the pressing step.

On the other hand, when compared these results with TPA analysis (where hardness, cohesiveness, and springiness significantly increased at higher levels of P/F ratios), might suggest that protein matrix had a major effect at large deformations which could not be determined efficiently at small deformations.

Microstructure

Pore size and porosity of the model fresh cheeses were analyzed using the images taken by Environmental Scanning Electron Microscopy (ESEM). For both pore size and porosity, the effect of formulation and homogenization pressure did not show significant differences ($p < 0.05$) (Figures 3.15 – 3.18). Also, samples were averaged by P/F and homogenization pressure to see the trends in porosity and pore size, although all samples were similar, samples processed at 0.9 and 2 P/F ratio seem to have lower porosities when compared to those processed at 1.3 and 1.7 P/F (Figure 3.19). It is known that pore size and porosity is related to the water holding capacity and permeability of milk gels which in turns affect the casein particles interactions that form the gel network (Lucey and others 2001). From micrographs, pores seemed very homogeneous at $\sim 0.5 \mu\text{m}$. This homogeneity might be caused by the application of the heat-induced acidification to form the curd, which has shown to be key for determine structural properties as explained previously. Also, utilizing 15 h of pressing might also have caused the same volume of whey expelled from the matrix regardless of the sample preparation conditions. Lucey and others (1998, 2000, 2001), studied the differences between heated and unheated milk gels, indicating that heated gels produced branched matrices with smaller and more defined pores compared to unheated gels, which had higher water-holding capacity and more interconnectivity of the network. Although no statistical differences were detected for porosity and pore size, qualitative some differences were observed for samples processed at higher levels of protein to fat ratios and homogenization pressures which seem to have formed smaller pores (Figure 3.20 – 3.23).

3.5 Conclusions

The physical properties and microstructure of a model fresh cheese were analyzed by Particle size, Texture Profile Analysis (TPA), Small Amplitude Oscillatory Shear (SAOS) rheology and imaging analysis (ESEM). The particle size of the cheese milk emulsion decreased significantly as protein to fat ratio (P/F) and homogenization pressure increased. For Texture Profile Analysis formulation had a major effect than homogenization pressure over TPA parameters, finding that hardness, cohesiveness, springiness and fracturability increased significantly with higher protein to fat ratios which are explained by more interactions between proteins due to the increased volume fraction of the protein matrix compared to fat which also can be related to higher surface area of the smaller particles formed as P/F increased. On the other hand, from the frequency sweeps tests, all samples followed a viscoelastic behavior showing higher levels of G' than G'' . Also, P/F ratio had a significant effect on $\tan\delta$, as P/F decreased $\tan\delta$ showed less relative viscous properties, suggesting a weaker gel formation; however this behavior needs further investigation. Microstructure analysis of porosity and pore size showed no significant differences between samples, which might be caused by the overnight pressing step, where reorientation of the curd granules was similar as well as the formation of small pores due to heat-induced acidification. In conclusion, the structure of a model fresh cheese made by heat-induced acidification was affected more prominently by formulation (P/F ratio) than by homogenization pressures, and the re-arrangement of protein micelles into a continuous aggregated matrix affected by heat and pH as well as the pressing step, seem to be a contributing factor for the microstructural conformation of the matrix under study. Microstructure of complex soft solids needs further investigation since many factors regarding constituents as well as processing, are interacting together to form the micro and macrostructural

characteristics and some of them may be more critical than others. Understanding those critical factors will help to create food structures with desired characteristics for targeted functional properties.

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3.7 Tables and Figures

Table 3.1 Formulation developed for model fresh cheese.

P/F	SMP (g)	AMF (g)	CaCl ₂ (g)	NaCl (g)	H ₂ O (g)	Solid content (%, w/w)	Total wt. (g)
0.9	280.85	106.52	0.27	33.75	1350	0.31	1771.39
1.3	306.81	80.56	0.27	33.75	1350	0.31	1771.39
1.7	322.59	64.78	0.27	33.75	1350	0.31	1771.39
2	330.89	56.48	0.27	33.75	1350	0.31	1771.39

SMP = Skim milk powder; AMF = Anhydrous milk fat.

Quantities of SMP and AMF were obtained considering the protein and fat content of each of the raw materials to reach 31% solid content (quantities suitable for microfluidization).

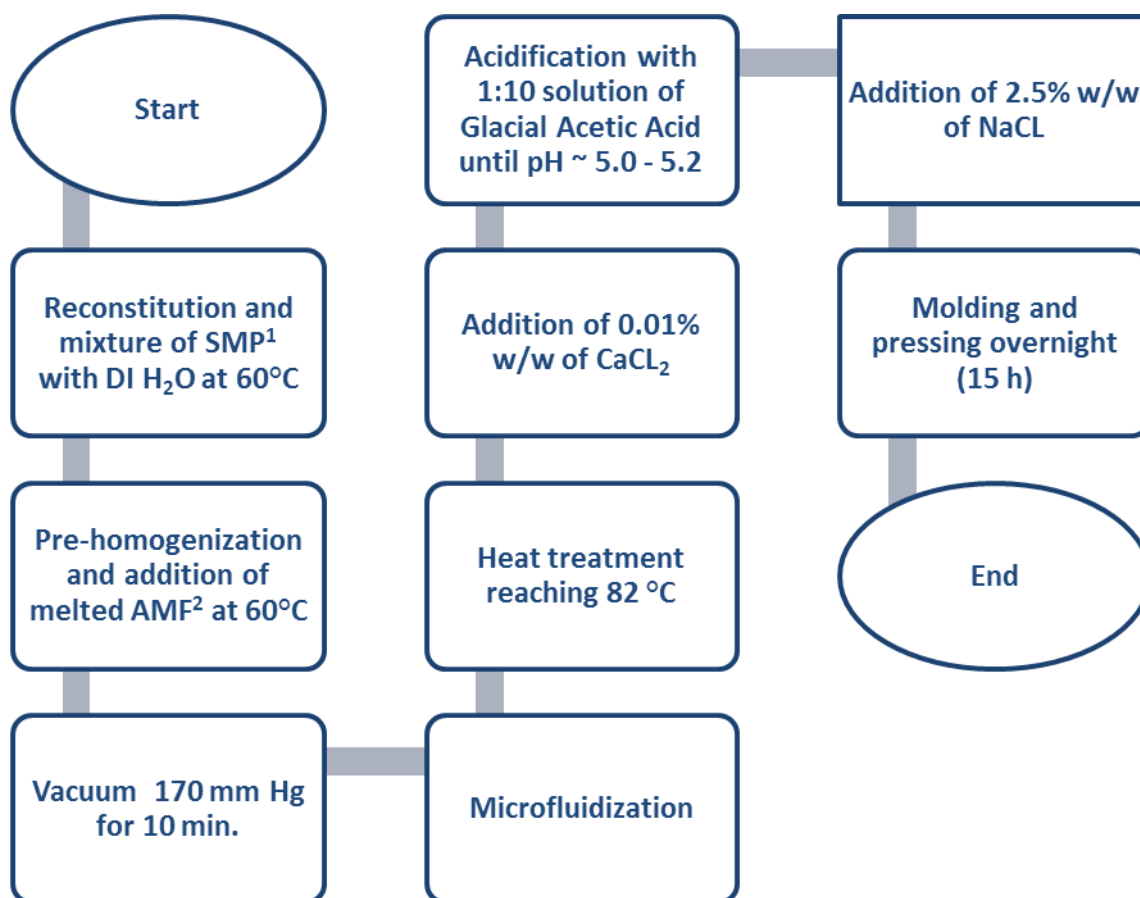
Table 3.2 Experimental design

Pressure (MPa)	Protein to fat ratio			
	0.9	1.3	1.7	2
17	P1R1	P1R2	P1R3	P1R4
50	P2R1	P2R2	P2R3	P2R4
75	P3R1	P3R2	P3R3	P3R4
150	P4R1	P4R2	P4R3	P4R4

P = Homogenization pressure

R = Protein to fat ratio

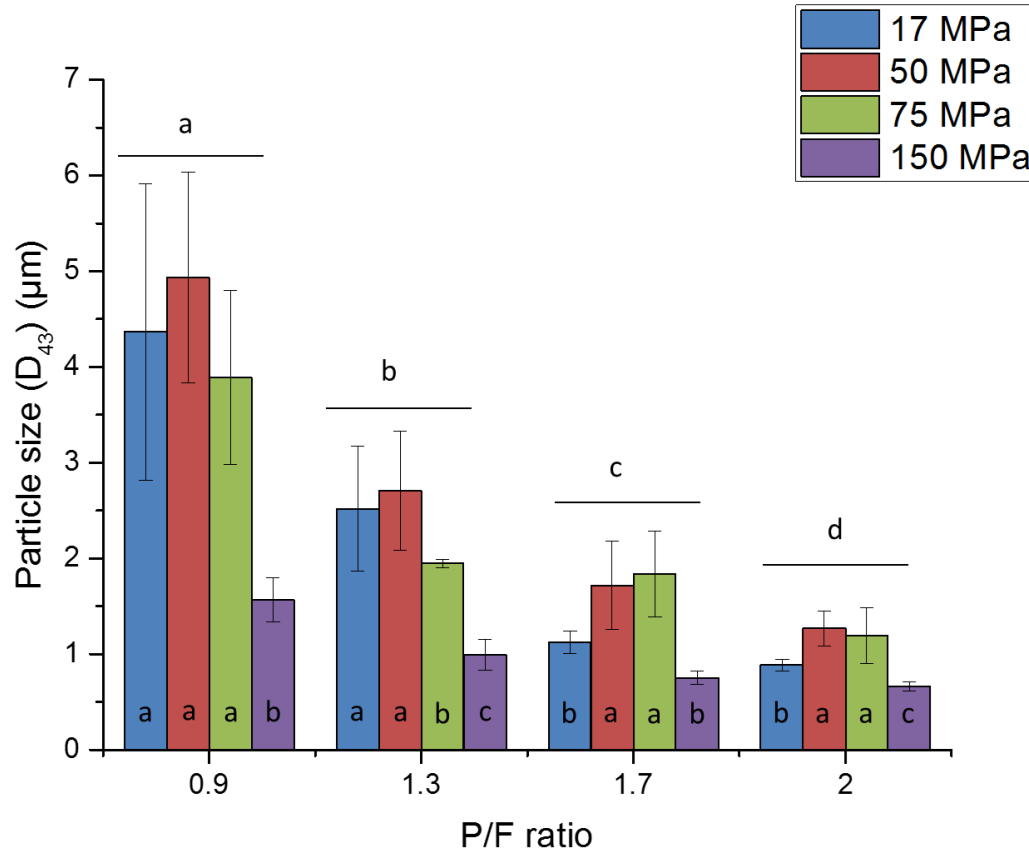
Figure 3.1. Model fresh cheese preparation process



¹SMP = Skim milk powder

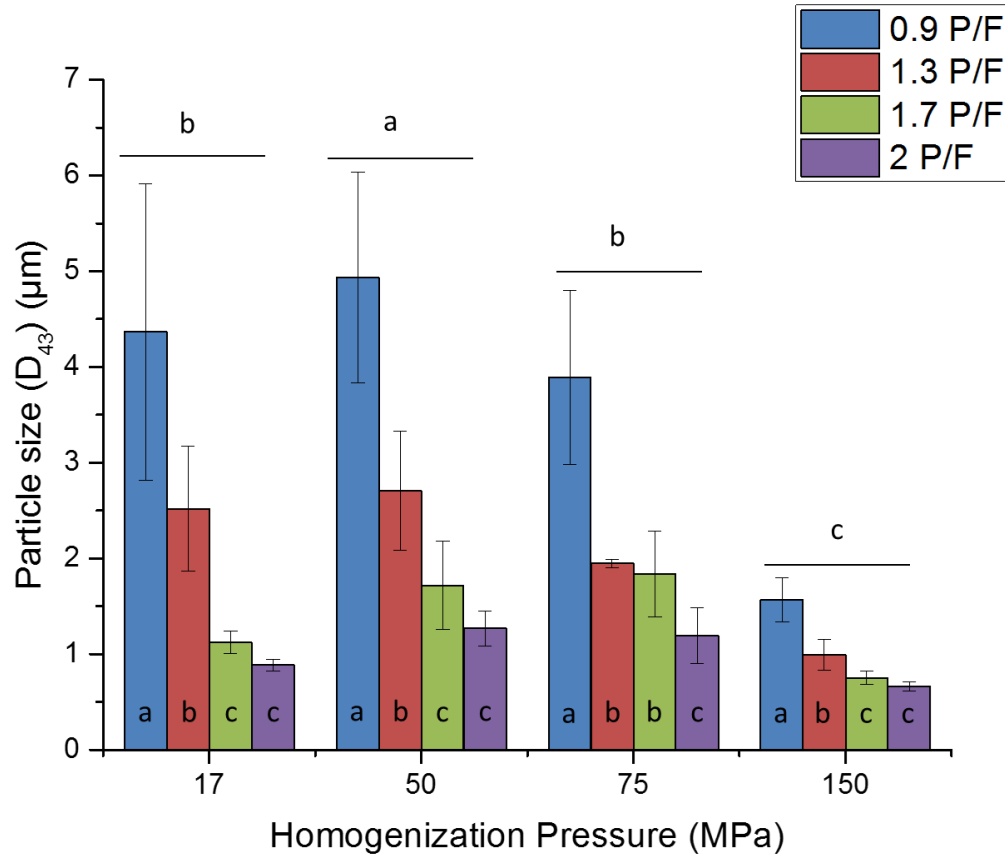
²AMF = Anhydrous milk fat

Figure 3.2 Particle size (d_{43}) of the cheese milk emulsion after microfluidization by protein to fat ratio.



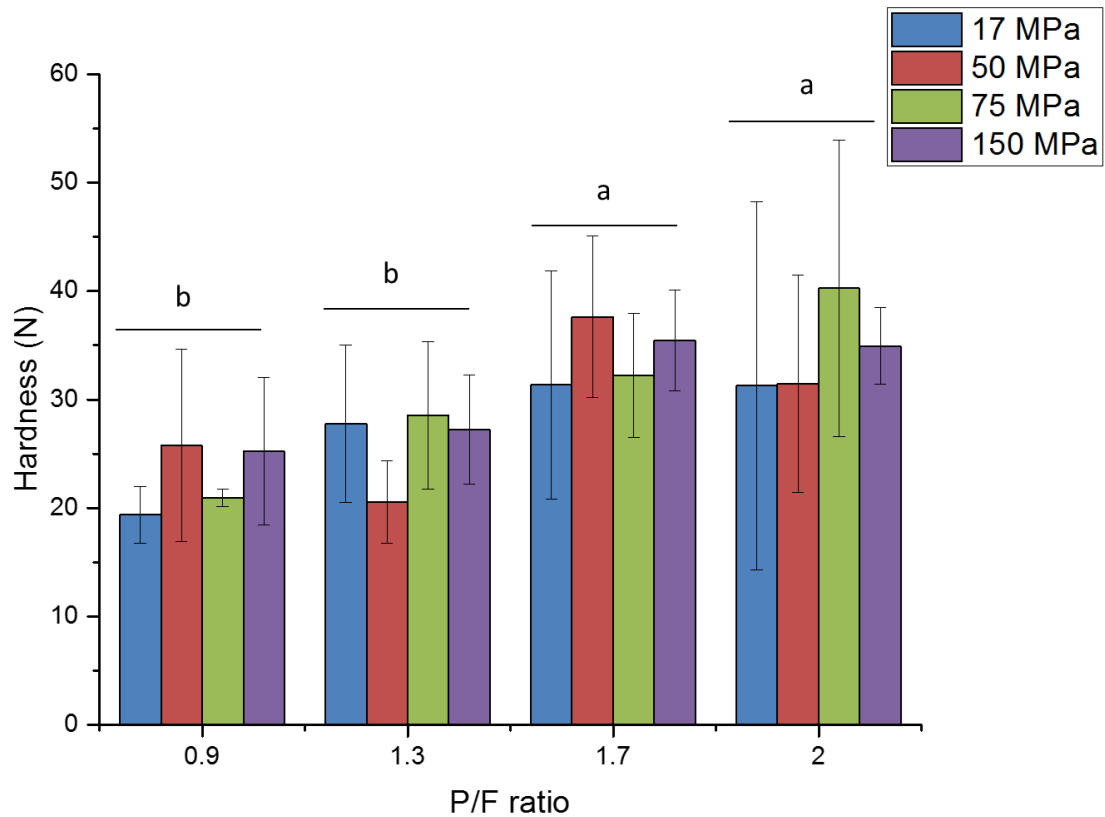
Particle size (d_{43}) in μm as a function of P/F (protein to fat ratio). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio and within each category processed at four levels of homogenization pressure. Protein to fat ratio had a significant effect ($p < 0.05$) in particle size (d_{43}) of the cheese milk emulsion.

Figure 3.3 Particle size (d_{43}) of the cheese milk emulsion after microfluidization by homogenization pressure.



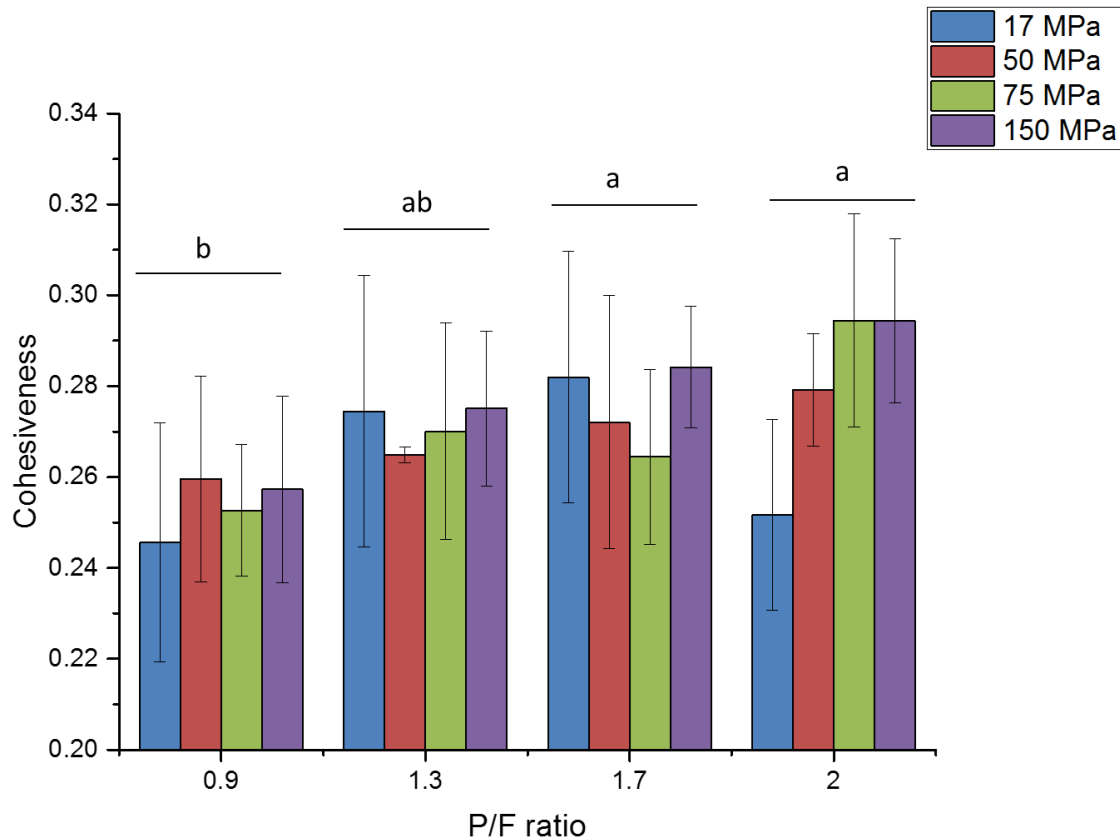
Particle size (d_{43}) in μm as a function of homogenization pressure. Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of homogenization pressure and within each category processed at four levels of protein to fat ratio. Homogenization pressure had a significant effect ($p < 0.05$) in particle size (d_{43}) of the cheese milk emulsion.

Figure 3.4 Hardness of the model fresh cheese samples obtained by Texture Profile Analysis



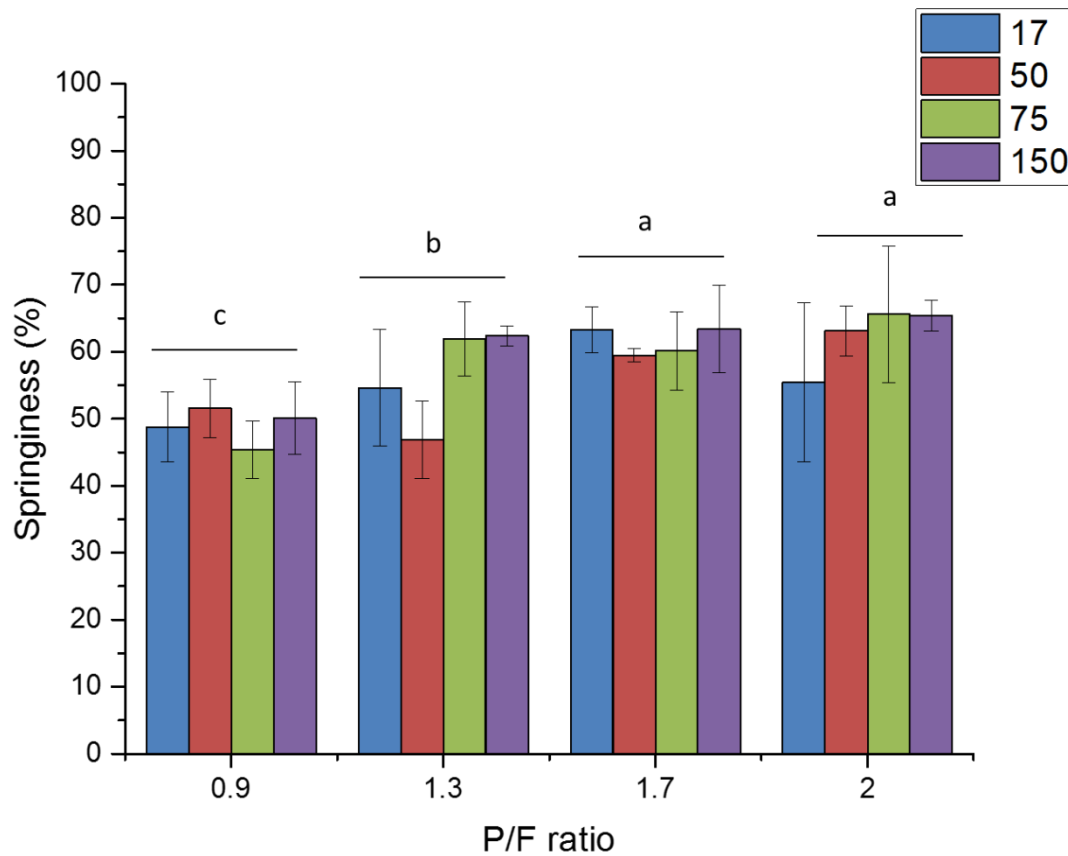
Hardness as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect ($p < 0.05$) in hardness of the model fresh cheese.

Figure 3.5 Cohesiveness of the model fresh cheese samples obtained by Texture Profile Analysis



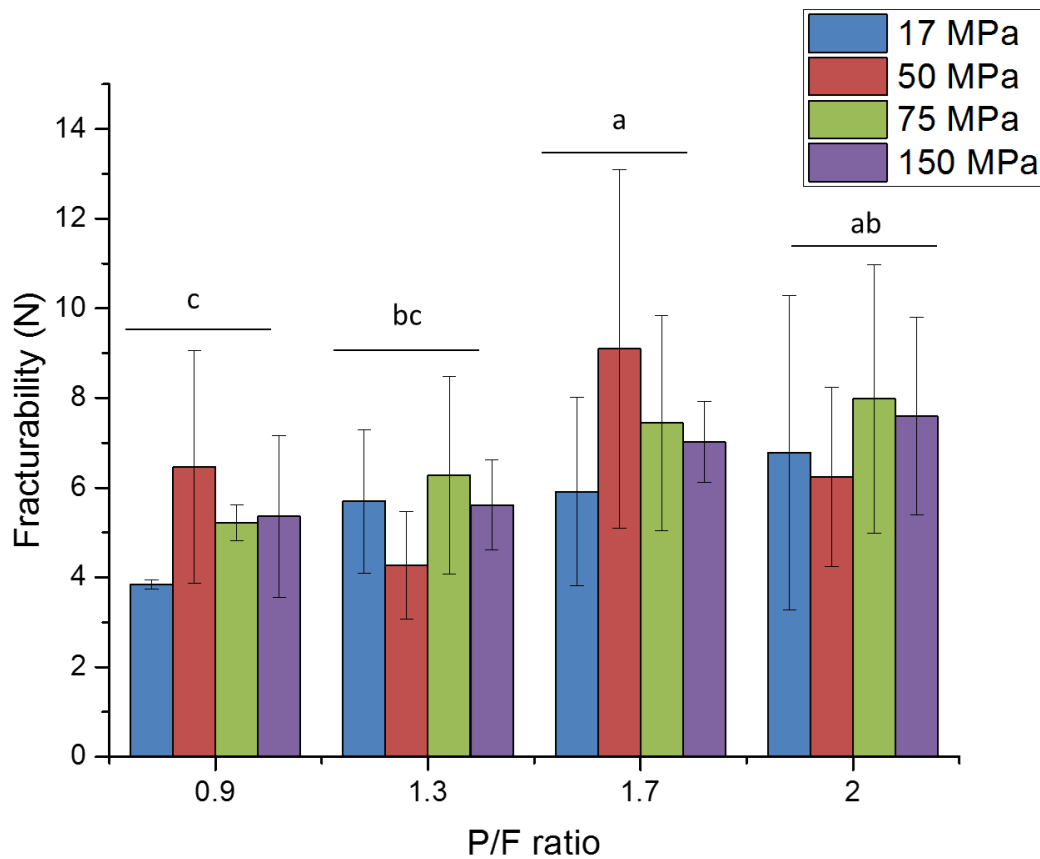
Cohesiveness as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect ($p < 0.05$) in cohesiveness of the model fresh cheese

Figure 3.6 Springiness of the model fresh cheese samples obtained by Texture Profile Analysis



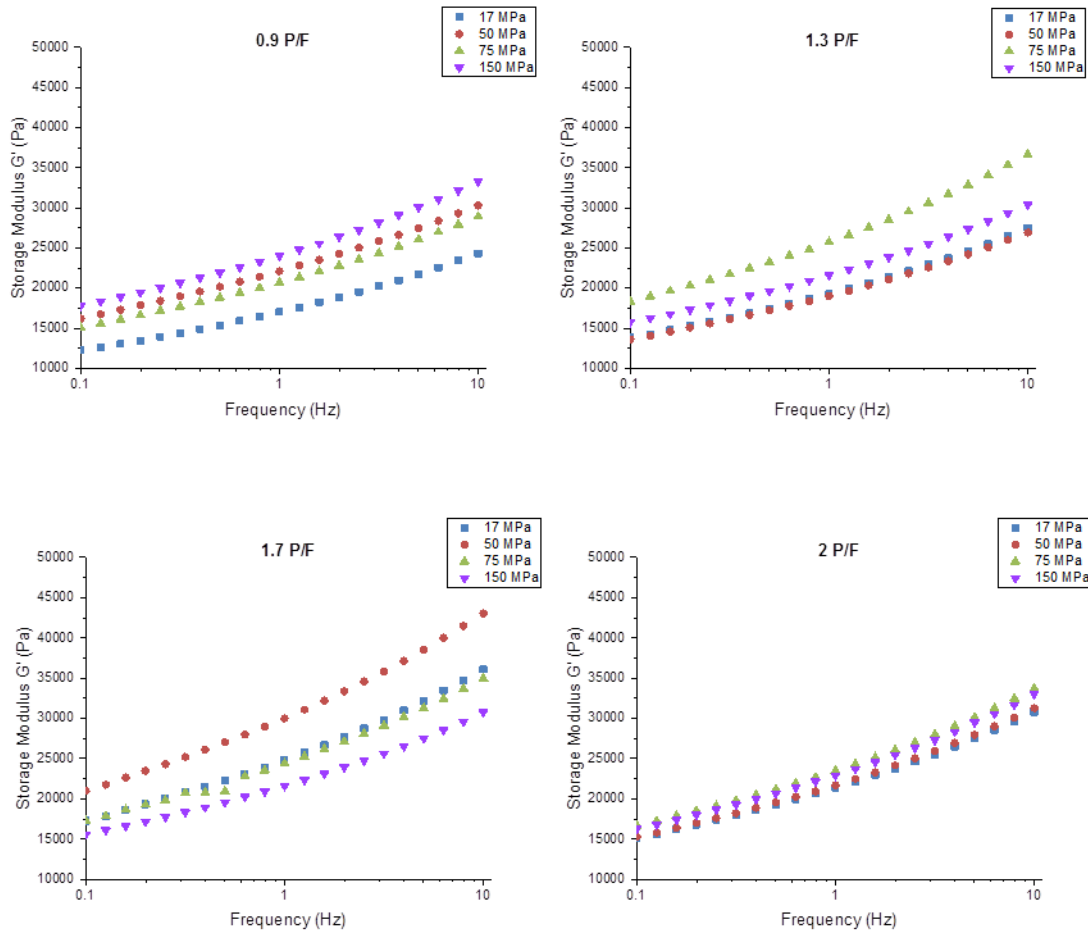
Springiness as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect ($p < 0.05$) in springiness of the model fresh cheese.

Figure 3.7. Fracturability of the model fresh cheese samples obtained by Texture Profile Analysis



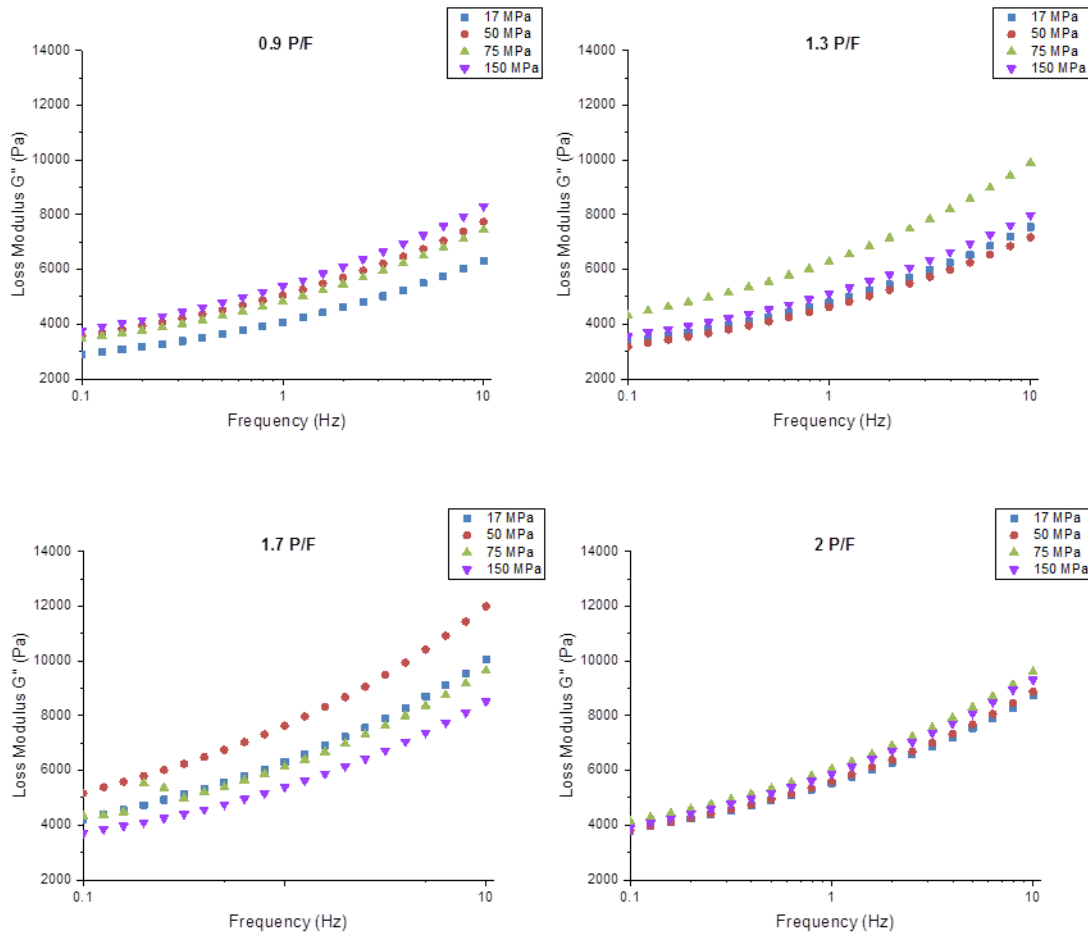
Fracturability as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect ($p < 0.05$) in Fracturability of the model fresh cheese

Figure 3.8 Storage modulus (G') for model fresh cheese samples at different protein to fat ratios



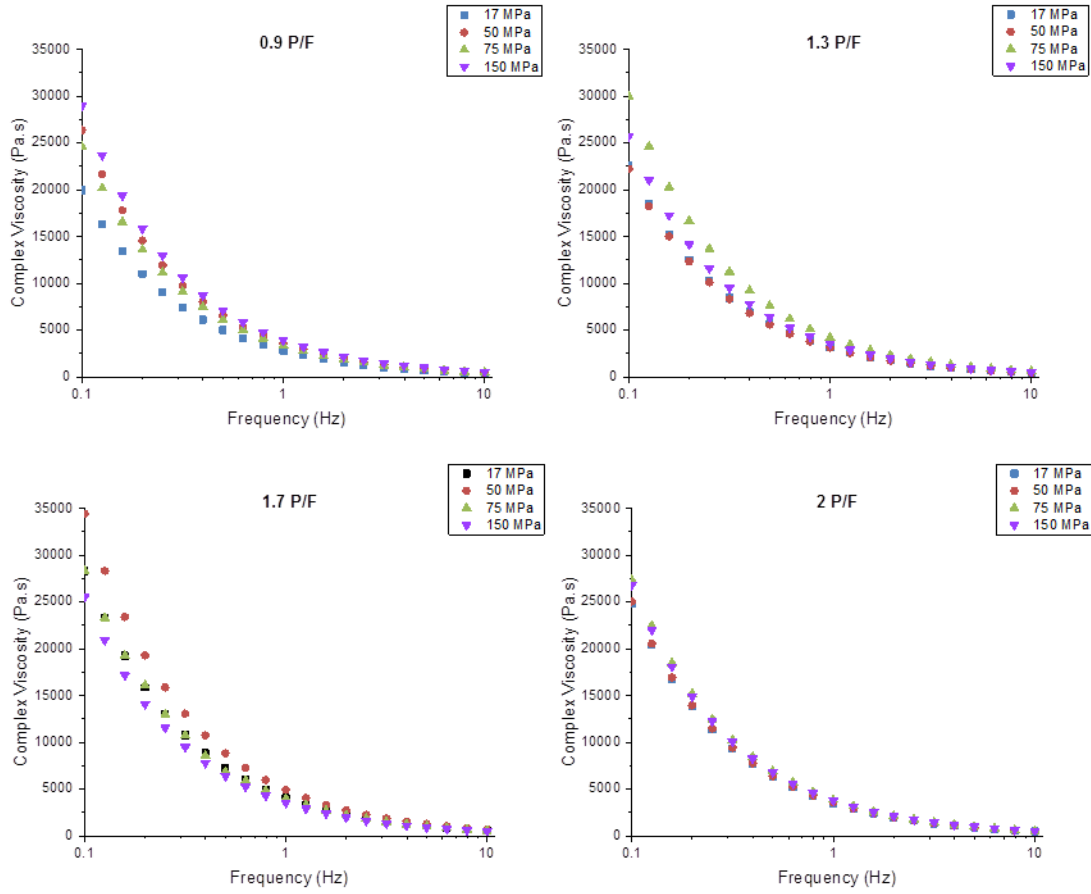
Storage modulus (G') spectra for model fresh cheese samples by different protein to fat ratio (P/F). Results expressed as mean ($n=3$). No clear trend was observed between samples processed at different homogenization pressures. At 2 P/F samples were more homogeneous.

Figure 3.9 Loss modulus (G'') for model fresh cheese samples different protein to fat ratios.



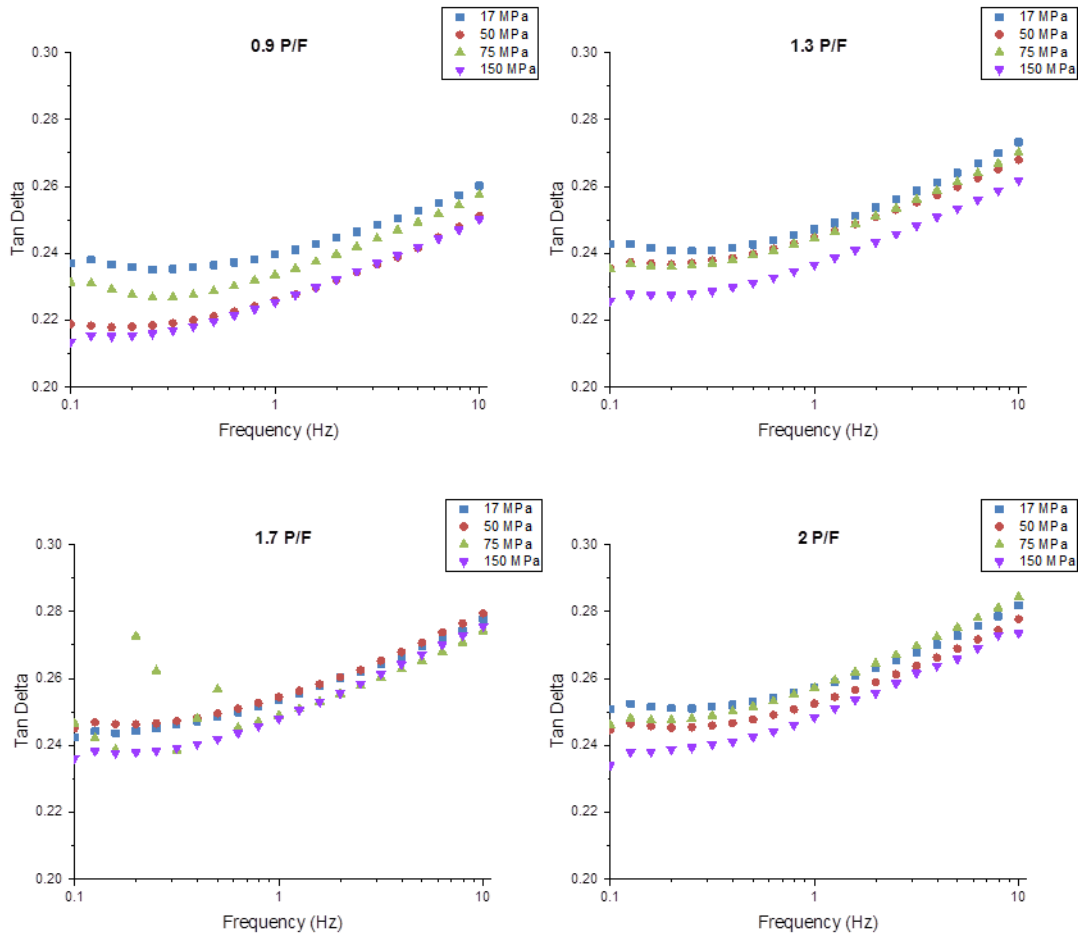
Loss modulus (G'') spectra for model fresh cheese samples by different protein to fat ratio (P/F). Results expressed as mean ($n=3$). No clear trend was observed between samples processed at different homogenization pressures. At 2 P/F samples were more homogeneous.

Figure 3.10 Complex viscosity for model fresh cheese samples by protein to fat ratio



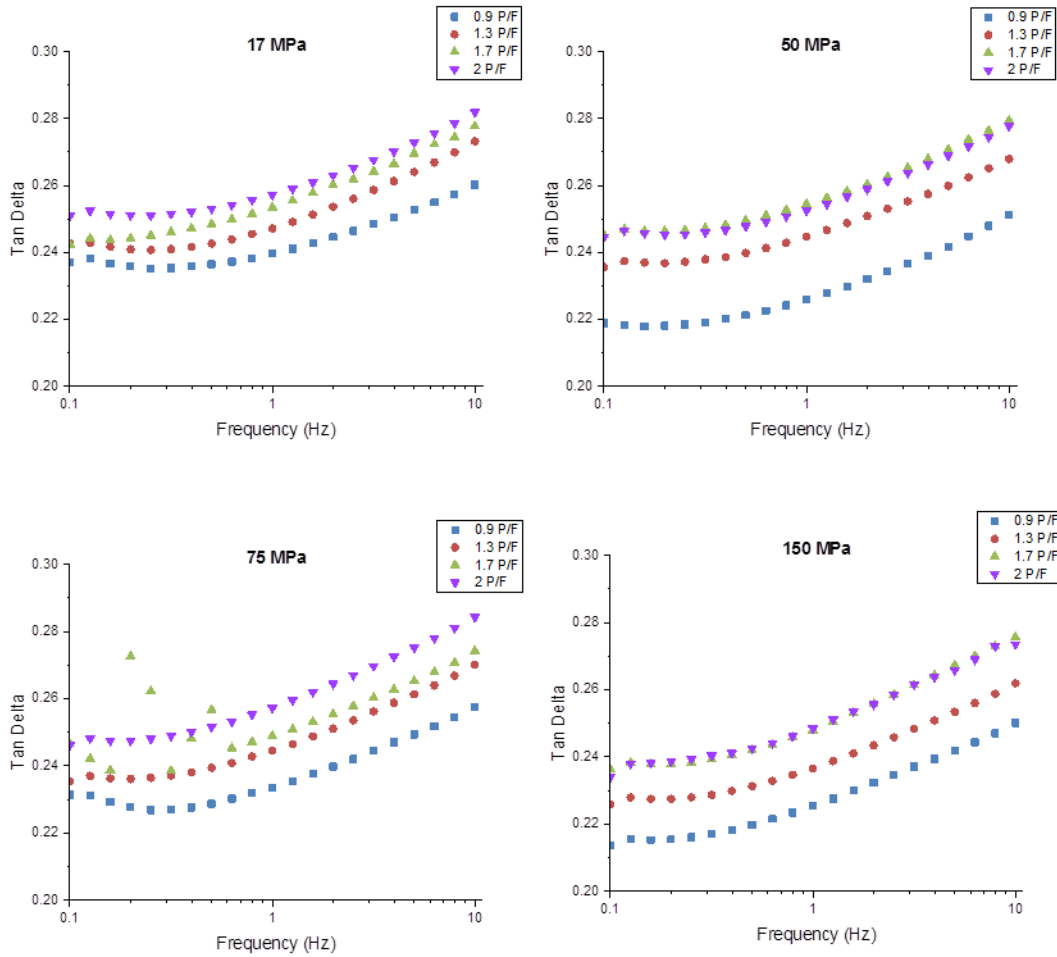
Complex viscosity (η) spectra for model fresh cheese samples by different protein to fat ratio (P/F). Results expressed as mean ($n=3$). No clear trend was observed between samples processed at different homogenization pressures.

Figure 3.11 $\tan \delta$ for model fresh cheese samples by protein to fat ratio.



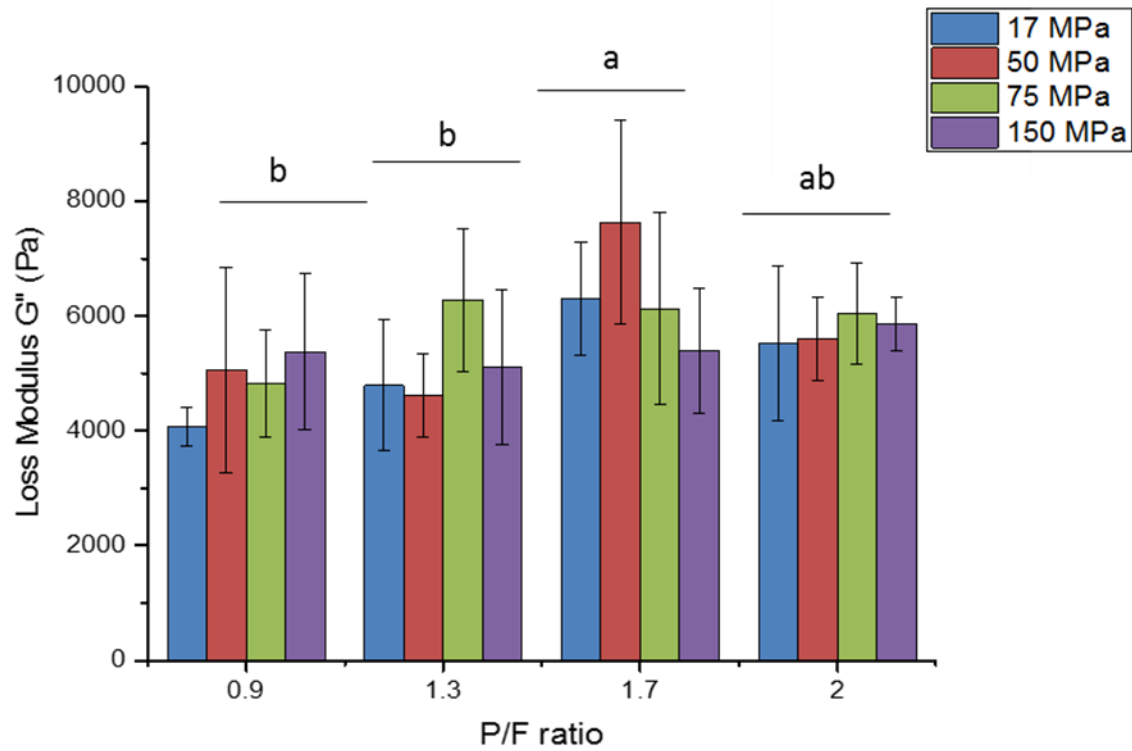
$\tan \delta$ spectra for model fresh cheese samples by different protein to fat ratio (P/F). Results expressed as mean ($n=3$). Samples processed at 0.9 P/F obtained lower $\tan \delta$ values.

Figure 3.12 Tan δ for model fresh cheese samples by homogenization pressure



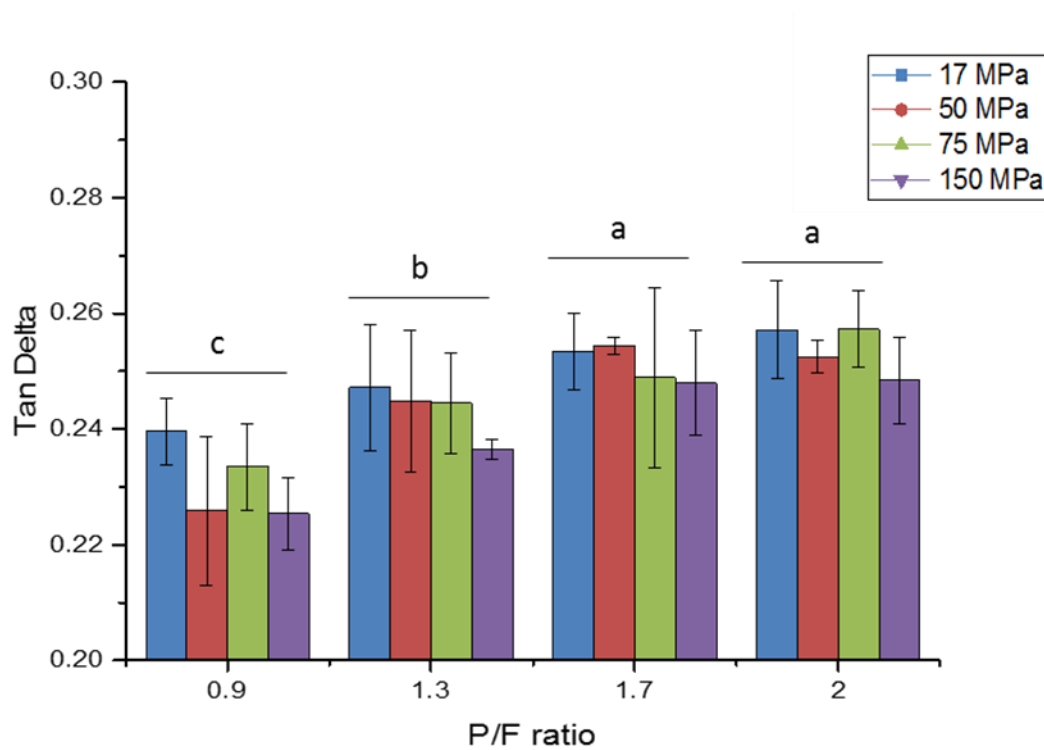
Tan δ spectra for model fresh cheese samples by different homogenization pressure (P/F). Results expressed as mean ($n=3$). A clearer trend can be seen within groups, where samples at 0.9 P/F obtained lower values of tan δ and samples at 2P/F obtained the highest values.

Figure 3.13 Statistical differences in loss modulus (G'') at frequencies of 1 Hz.



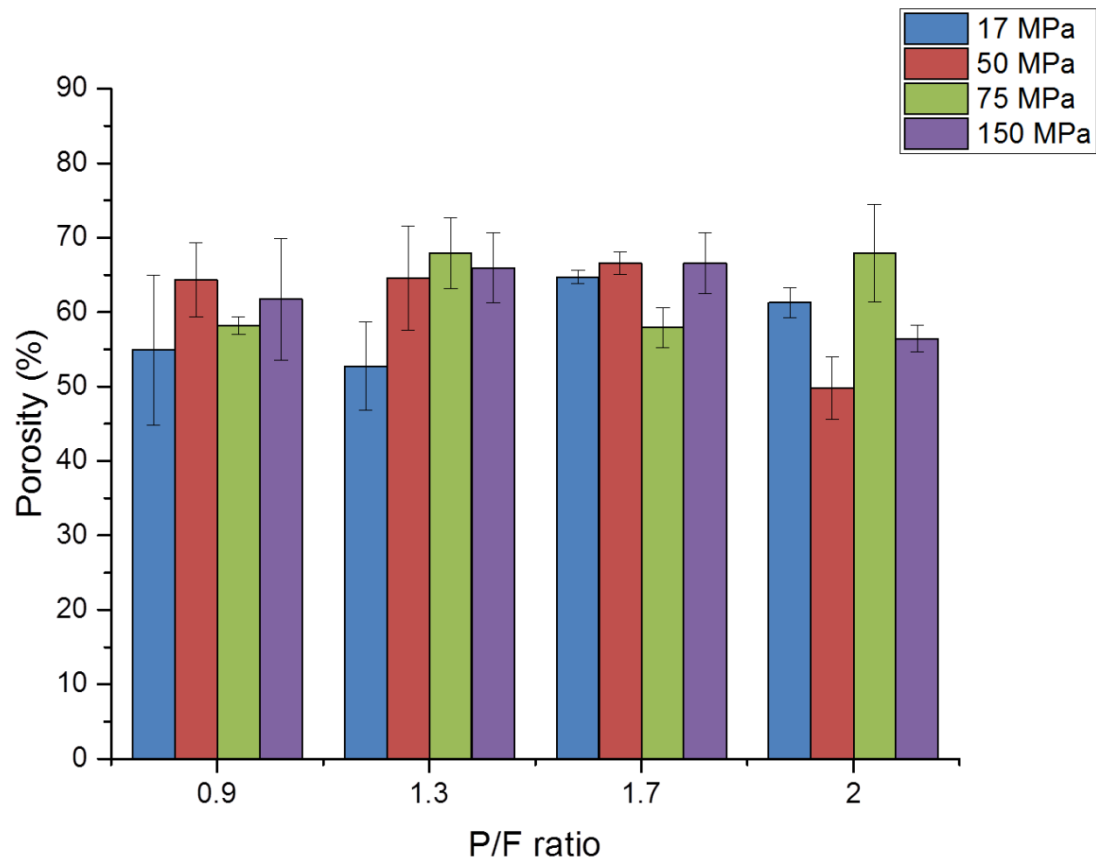
Loss modulus (G'') as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect (<0.05) in G'' of the model fresh cheese

Figure 3.14 Statistical differences in $\tan \delta$ at frequencies of 1 Hz.



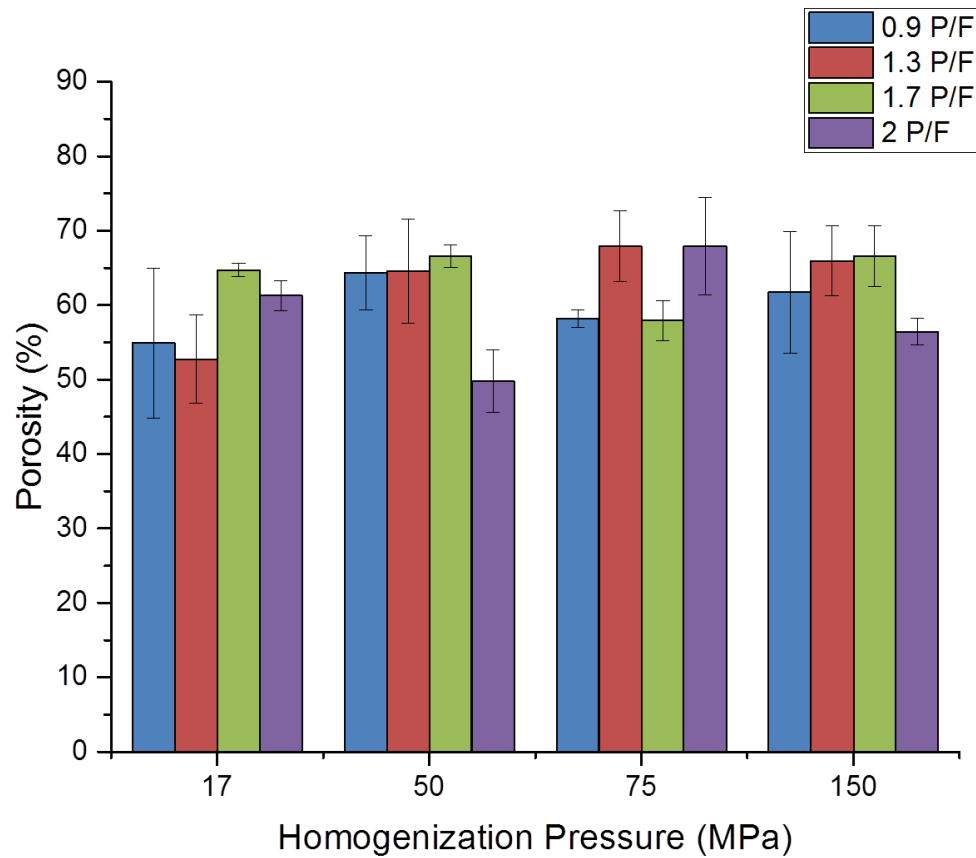
$\tan \delta$ as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). Statistical differences are denoted by different letter for each level of protein to fat ratio. Bars within groups did not differ significantly. Formulation had a significant effect ($p < 0.05$) in $\tan \delta$ of the model fresh cheese.

Figure 3.15 Porosity by protein to fat ratio



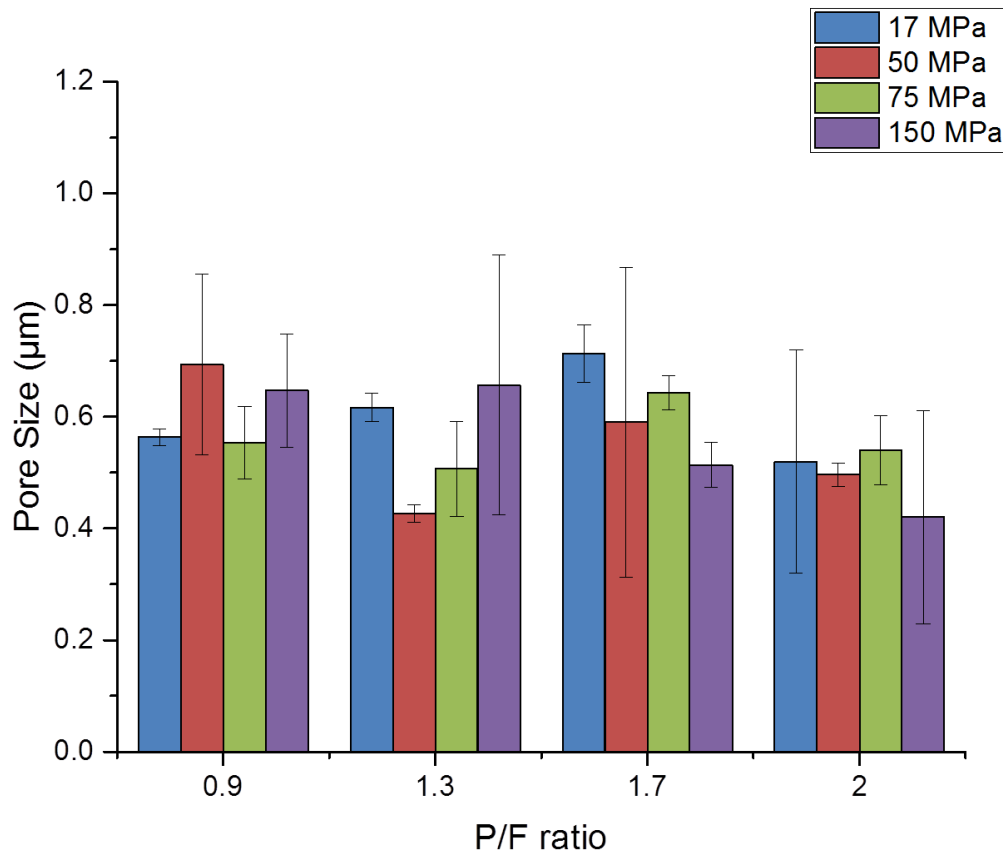
Porosity as a function of protein to fat ratio (P/F). Results expressed as mean \pm standard deviation ($n=3$). No statistical differences ($p < 0.05$) were found for porosity analyzed by P/F ratio.

Figure 3.16 Porosity by homogenization pressure



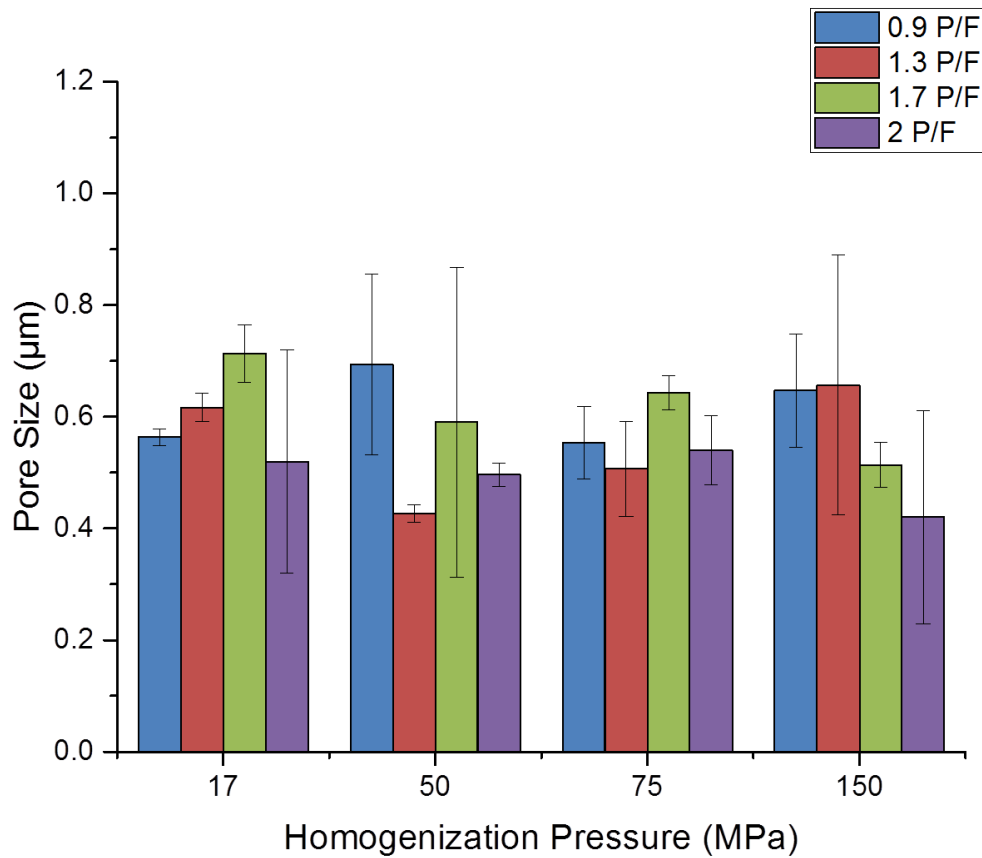
Porosity as a function of homogenization pressure. Results expressed as mean \pm standard deviation ($n=3$). No statistical differences ($p < 0.05$) were found for porosity analyzed by homogenization pressure.

Figure 3.17 Pore size by protein to fat ratio



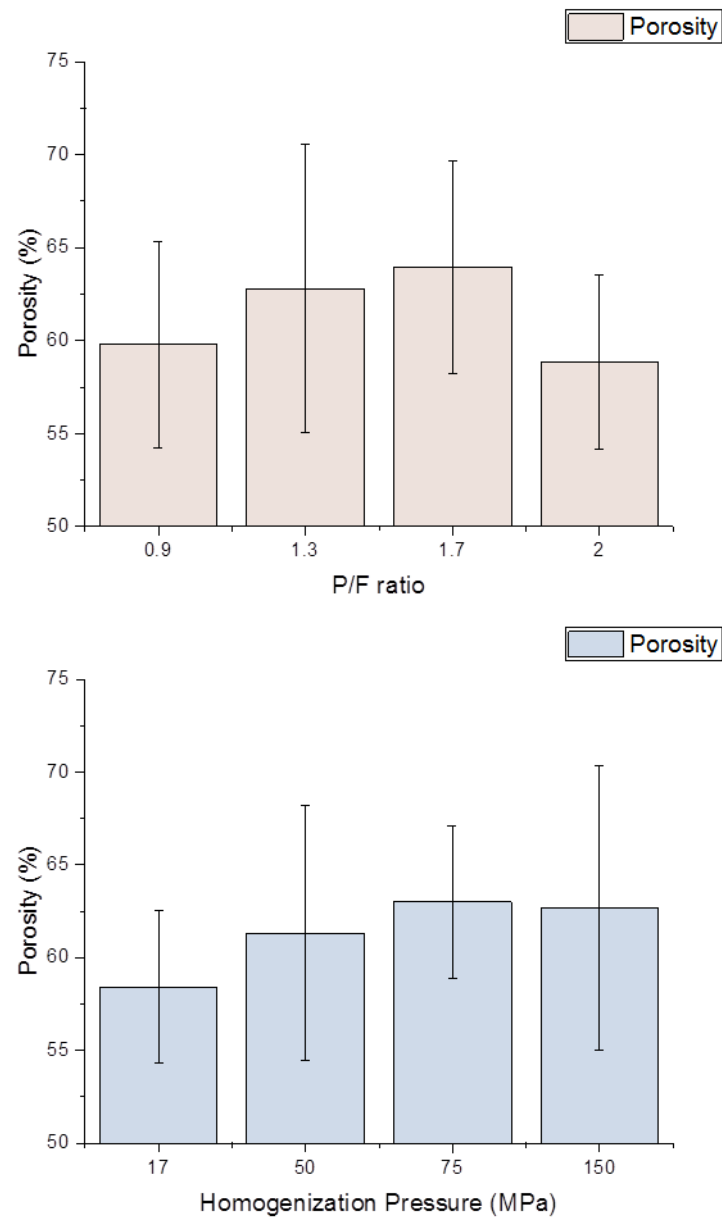
Pore size as a function of protein to fat ratio P/F. Results expressed as mean \pm standard deviation ($n=3$). No statistical differences ($p < 0.05$) were found for pore size analyzed by P/F

Figure 3.18 Pore size by homogenization pressure.



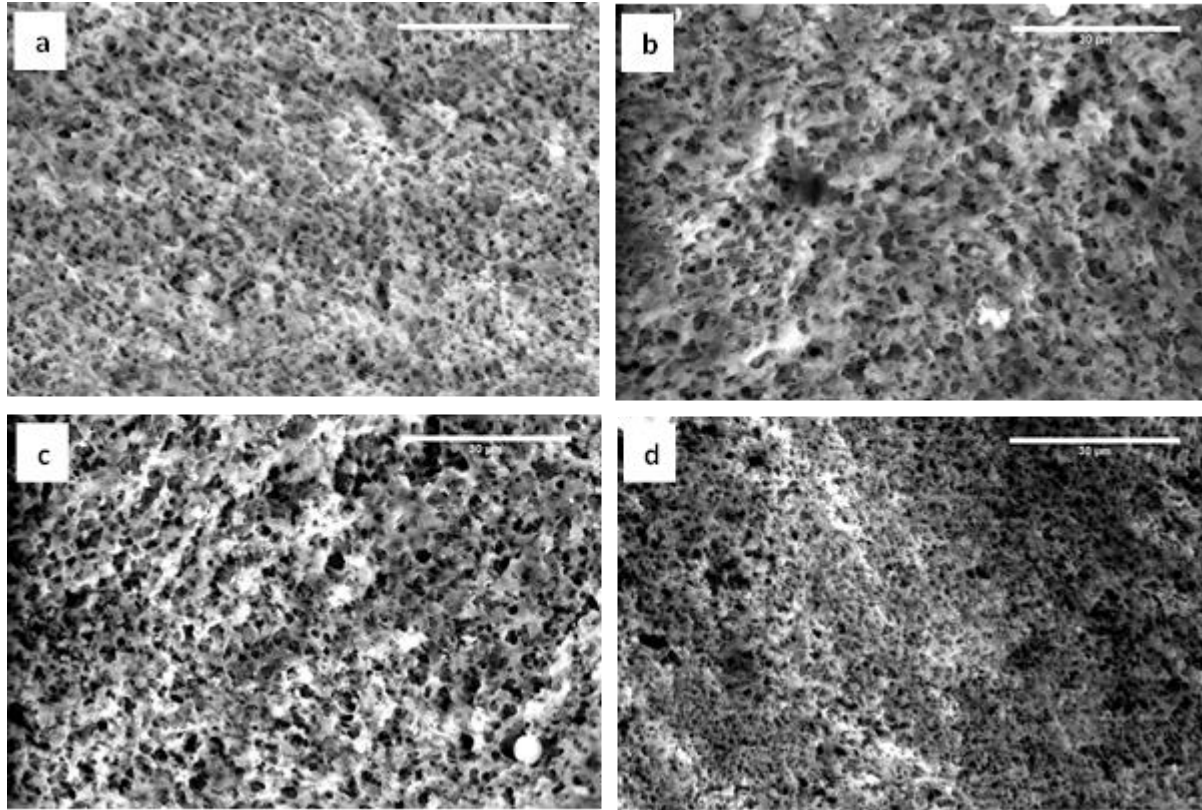
Pore size as a function of homogenization pressure. Results expressed as mean \pm standard deviation ($n=3$). No statistical differences ($p < 0.05$) were found for pore size analyzed by homogenization pressure.

Figure 3.19 Porosity averaged by protein to fat ratio and homogenization pressure



Porosity averaged by protein to fat ratio P/F and homogenization pressure. Results expressed as mean \pm standard deviation ($n=3$). Even though no significant differences were found for the two main effects, porosity seem to decrease at the two extremes of P/F (0.9 and 2) and homogenization pressure (17 and 150 MPa)

Figure 3.20 ESEM images processed at 17 MPa and different protein to fat ratios



ESEM micrographs at 2000 x processed at 17 MPa. a) 0.9, b) 1.3, c) 1.7 and d) 2 P/F ratios respectively. Scale bar at 30 μm

Figure 3.21 ESEM images processed at 50 MPa and different protein to fat ratios

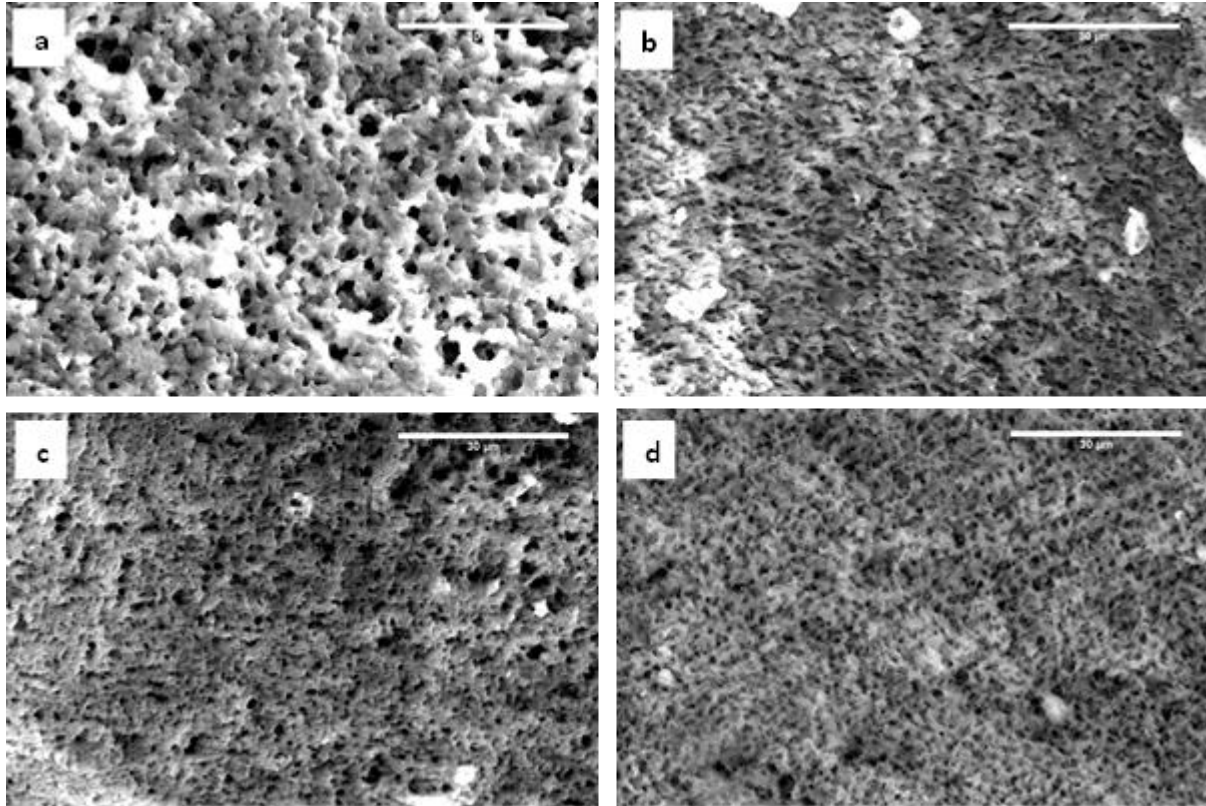
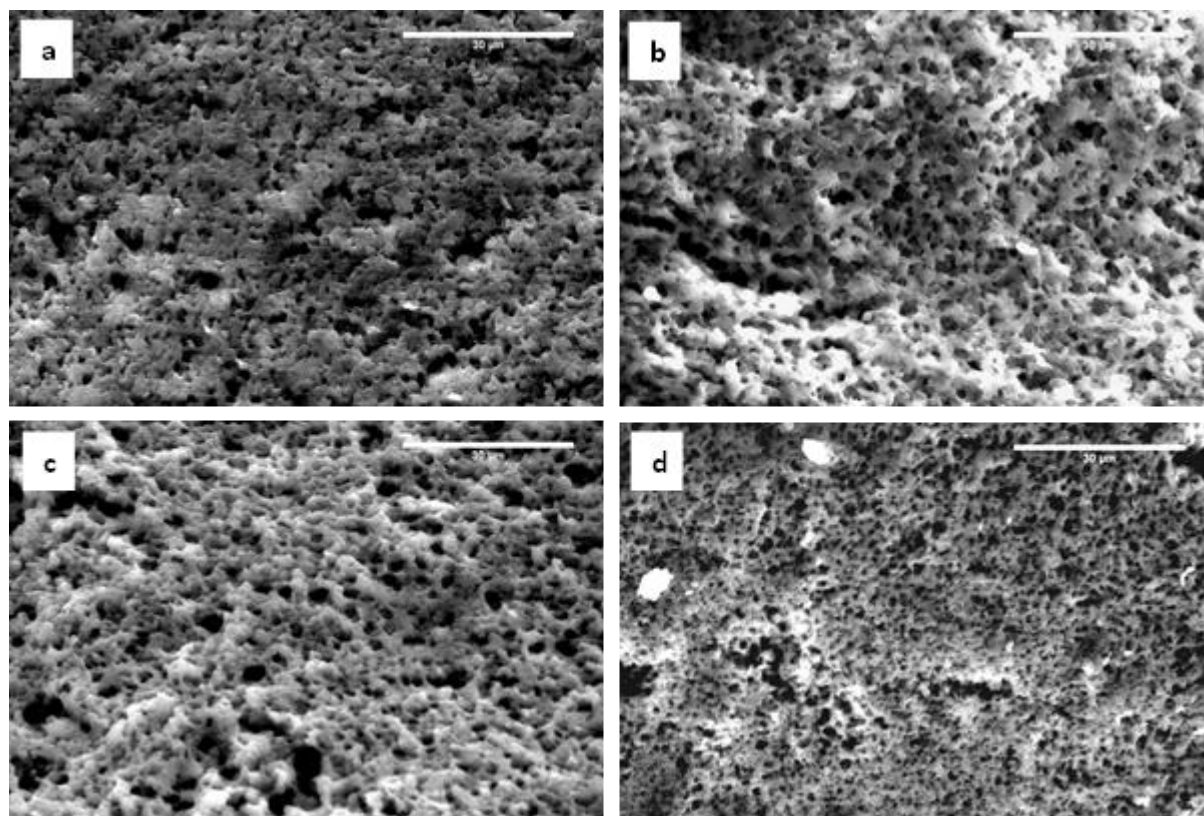
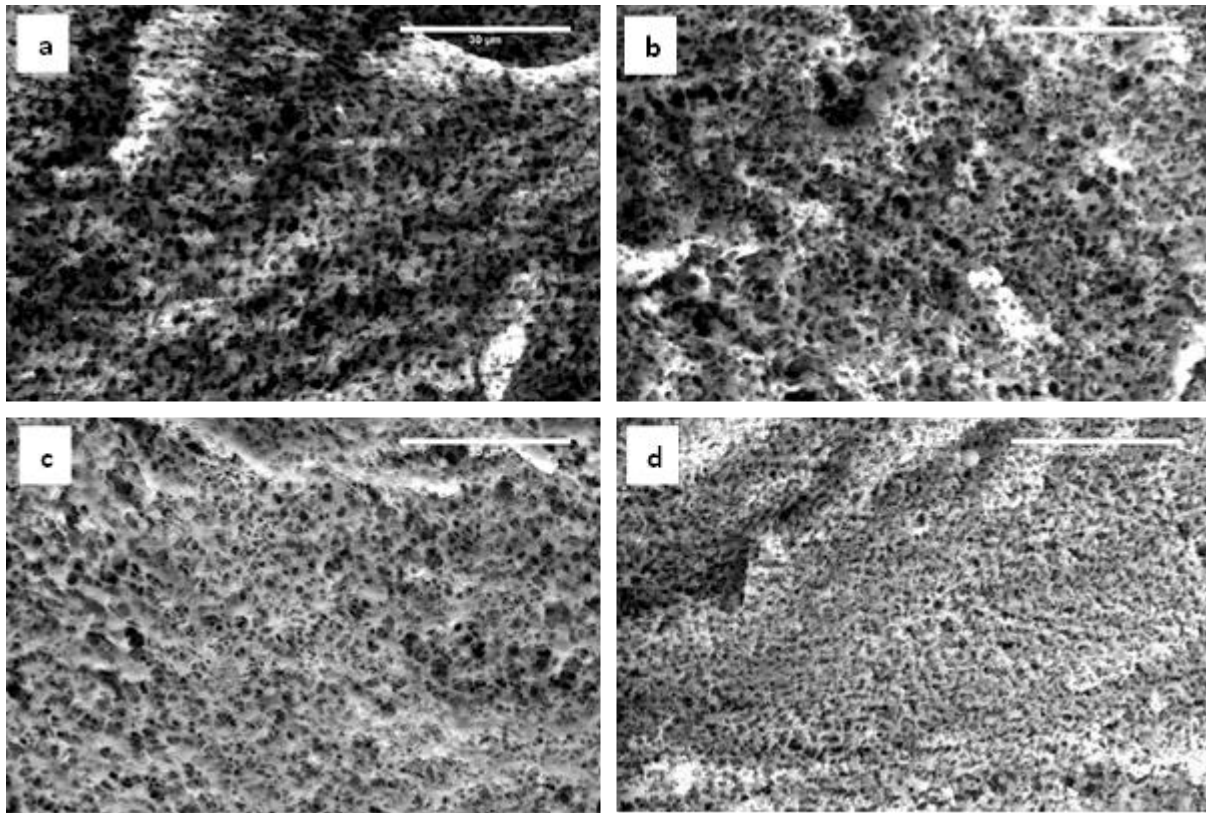


Figure 3.22 ESEM images processed at 75 MPa and different protein to fat ratios



ESEM micrographs at 2000 x processed at 75 MPa. a) 0.9, b) 1.3, c) 1.7 and d) 2 P/F ratios respectively. Scale bar at 30 µm.

Figure 3.23 ESEM images processed at 150 MPa and different protein to fat ratios



ESEM micrographs at 2000 x processed at 150 MPa. a) 0.9, b) 1.3, c) 1.7 and d) 2 P/F ratios respectively. Scale bar at 30 μm .

Chapter 4. Bioaccessibility of vitamin D₃ in a model fresh cheese affected by protein to fat ratio and homogenization pressure

4.1 Abstract

Bioaccessibility of vitamin D₃ was analyzed in a fortified soft solid matrix using a model fresh cheese as an example. Samples were processed at different formulations (protein to fat ratio) and homogenization pressures using a microfluidizer. It was hypothesized that increasing protein to fat ratio and homogenization pressure would increase the interfacial surface area of the fat particles in the model fresh cheese improving the rates of digestion and thus make vitamin D₃ more bioaccessible. Three samples representing the lowest and highest protein to fat ratios (0.9 and 17) and homogenization pressures (17 and 150 MPa) were selected from the experiment conducted in chapter 3 to evaluate the relationship between the structural characteristics and bioaccessibility. The cheese milk was fortified with a solution of crystalline vitamin D₃ at a concentration of 2.5 µg/mL. After cheese formation, extraction of vitamin D₃ was performed using saponification and solvent extraction. Bioaccessibility was analyzed using a two stage *in vitro* digestion followed by solvent extraction and the percentage of vitamin D₃ recovery after digestion was recorded through HPLC. Vitamin D₃ bioaccessibility was reduced by increasing P/F ratio and homogenization pressure, suggesting that bioaccessibility in this matrix is not dictated completely by the fat particle surface area but by the coexistence of the two main phases (fat and protein) integrating the matrix. Higher P/F ratios and homogenization pressures may have caused stronger protein-protein interactions and thus a decrease on protein hydrolysis during digestion, which resulted in decreased release of the fat soluble vitamin D₃.

4.2 Introduction

The food structure is a relevant property to consider when a new product is developed with specific functional properties including the improvement of the nutritional value. Although information about nutritional content of food products is currently available in the nutritional facts of labelled products, the amount that is absorbed after digestion is not clear. It is believed that several factors interacting in the food matrix might affect the release of nutritional components in processed foods (Parada and Aguilera 2007). Many studies have suggested a significant relationship between food matrix, digestion kinetics and mechanisms of nutrient absorption (Marze 2013; Le Feunteun and others 2014; Gallier and others 2014). For these studies, *in vivo*, and *in vitro* digestion models have been utilized to gain a better understanding of this relationship. *In vitro* models have the advantage of being less expensive and getting rapid results compared to *in vivo* models, however, complete physiological factors related to digestion are not taken into account. *In vitro* models have been adopted to study bioaccessibility of several nutrients, which by definition is the amount of a compound that is released out of the food matrix and becomes available for absorption (Fernández-García and others 2009). Most of the studies found in literature targeting the relationship of food matrix and micronutrient bioaccessibility were conducted with raw and processed fruits or vegetables. There are limited number of studies using more complex food matrices such as soft solids which are described as food matrices that show solid and liquid-like properties, and that can be found in several products such as jellies, processed meats, and dairy products.

Cheese is a complex nutrient-dense dairy product (Fox and McSweeney 2004), considered as a good carrier for fat-soluble micronutrients such as vitamin D (Kazmi and others 2007; Ganesan and others 2011; Tippetts and others 2012), which is important for bone

formation. The diversification of fortified products with vitamin D is important due to deficiencies found in many countries around the world. It has been demonstrated that the concentration, stability, and bioaccessibility of micronutrients in cheese products is influenced by the structure of the food matrix, its components and processing parameters (Öste and others 1997; O'Brien and O'Connor 2004). The objective of this study was to analyze the bioaccessibility of vitamin D₃ (using *in-vitro* digestion) in a fortified model fresh cheese as an example of a soft-solid matrix, processed at different formulations (protein to fat ratio) and homogenization pressures using the microfluidizer. It was hypothesized that increasing protein to fat ratio and homogenization pressure would increase the interfacial surface area of fat particles (Marze 2013) in the model fresh cheese, improving rates of digestion and thus make vitamin D₃ more bioaccessible.

4.3 Materials and Methods

Determination of standard concentration and development of standard curve

To determine the standard concentration, a primary stock solution of vitamin D₃ was prepared. 2 mg of vitamin D₃ (crystalline USP, Sigma-Aldrich, INC., Milwaukee, WI., U.S.A) were dissolved in 10 mL ethanol (EtOH denatured for HPLC, Sigma-Aldrich, INC., Milwaukee, WI) to obtain a concentration of 0.2 mg/mL. The stock solution was diluted 10x to obtain a secondary stock solution to a final concentration of 20 µg/mL (appropriate for spectrophotometer measurements). The exact concentration of the stock solution was determined by its extinction coefficient at 260 nm for cholecalciferol in ethanol ($E^{1\%} = 475$) (Barba and others 2011). Five replicates of 1 mL from the secondary stock solution were filtered in 0.45 µm PTFE (Macherey-Nagel Inc., Bethlehem, PA. U.S.A.) and analyzed in a GENESYS 10S UV – Vis

Spectrophotometer (Fisher Scientific Company LLC., Hanover Park, IL. U.S.A.) at 260 nm. Pure ethanol was used as blank. Absorbance was measured procuring a coefficient of variation <5%. The standard concentration of the stock solution was calculated by the Beer's law formula (Eq.3) which relates the absorbance with the extinction coefficient of the compound of interest at a certain wavelength. The standard concentration was used to generate the standard curve for the HPLC using diluted vitamin D₃ primary stock solution. The standard curve was built at vitamin D₃ concentrations of 0.1, 0.5, 1, 5, and 10 µg/mL using 100% Methanol HPLC grade (Fisher Scientific Company LLC., Hanover Park, IL) as a dilution solvent as well as a mobile phase. Concentrations were injected in duplicate. The stock solutions were kept under dark conditions at -20°C.

$$C \text{ (g/100 mL)} = \frac{A}{E^{1\%}} \quad [\text{Eq.3}]$$

C = Concentration in g/100 mL

A = Absorbance

E^{1%} = Extinction coefficient 1% in EtOH at 260 nm

Fortification of model fresh cheese with vitamin D₃

Vitamin D₃ fortification of model fresh cheese was performed using the method proposed by Kazmi and others (2007). A solution of vitamin D₃ was made by dissolving 25 mg of crystalline vitamin D₃ in 10 mL of ethanol to reach a final concentration of 2.5 mg/mL or 100000 IU vitamin D₃/mL. This stock solution was kept under dark conditions at -27°C.

For model cheese fortification, an aliquot (1.8 mL) of the stock solution was added to the melted anhydrous milk fat to reach a concentration of 2.5 µg of vitamin D₃/mL of milk (Kazmi and others 2007; Wagner and others 2008) (this calculation was based on ~1771.3 g of milk produced in each batch before microfluidization), and mixed using a KA T-25 Digital High-

Speed Homogenizer (IKA Works Inc., Wilmington, N.C., U.S.A.) at 5000 rpm for 2 minutes. Immediately, the fortified milk fat was added to the reconstituted skim milk following the method for making model fresh cheese explained in detail in chapter three (Figure 3.1). Subdued light conditions were used during fortification and processing. Three experimental units were chosen from the main experimental design for vitamin D₃ fortification (P1R1, P1R4, P4R4, Table 3.2) in order to have a general view of the effects of the samples processed at highest and lowest protein to fat ratios and homogenization pressures.

Vitamin D₃ extraction and quantification from fortified milk, cheese and whey

Before *in vitro* digestion analysis, extraction of vitamin D₃ from cheese milk, model cheese and whey expelled during curd formation was performed. The saponification method reported by Leskauskaite and Jasutiene (2016) was used with some modifications. 2 g of sample (milk, cheese or whey) was weighed into a 20 mL glass vial. For cheese samples, additional preparation was required before saponification, few drops of DI water were added to the cheese sample and mixed in a mortar and pestle (2 drops of water for each gram of cheese) to aid the vitamin D₃ extraction (Kazmi and others 2007). Subsequently, 8 mL of ethanol (Decon Labs. Inc. King of Prussia, PA., U.S.A.); 0.2 g ascorbic acid (Sigma-Aldrich, INC., Milwaukee, WI.), a spatletip of pyrogallol ACS 99% (Fisher Scientific Company LLC., Hanover Park, IL., U.S.A.) and 3 mL of 50% aqueous Potassium Hydroxide ACS solution (Fisher Scientific Company LLC., Hanover Park, IL.) were added to the vial. The vial was purged with a nitrogen stream to avoid oxidation, covered with aluminum foil and left it overnight (15 h) under orbital shaking in an incubator (Incu-Shaker mini, Benchmark Scientific, Inc. Edison, NJ., U.S.A.) at room temperature and 95 rpm. On the next day, a solvent extraction was performed. Each sample was

transferred to a 50 mL conical falcon tube where 5 mL of hexane and 2 mL of double deionized H₂O were added. The mixture was vortexed for 2 min and centrifuged (Sorvall ST 16 R, Fisher Scientific Company LLC., Hanover Park, IL) at 2500x g for 5 min at 20°C. After centrifugation, supernatant was collected, and extraction was repeated twice. The collected supernatant was evaporated using a nitrogen stream, reconstituted in 1 mL methanol (Fisher Scientific) and filtered through a 0.45 µm PTFE (Macherey-Nagel Inc.). Samples were injected into reverse phase HPLC for vitamin D₃ quantification (Waters e2695 Separation Module, Waters, Milford, MA, U.S.A.) with a C18 gravity column (Nucleodur 3µm, 150 x 4 mm, Macherey-Nagel Inc.) and photo diode array detector (Waters PDA 996) at 265 nm. 100% HPLC-grade methanol (Fisher Scientific) was used as mobile phase at a flow rate 0.5 mL min⁻¹ and an injection volume of 20 µg/mL. Samples were measured in triplicate.

In vitro analysis and quantification of vitamin D₃ in HPLC

A two phase dynamic *in vitro* digestion (gastric and intestine) was used to analyze vitamin D₃ bioaccessibility in the model fresh cheese samples. The *in vitro* method utilized was an adaptation from the methods described by Miller and others (1981), Garret and others (1999) and Etcheverry and others (2012) for the analysis of nano-emulsions fortified with vitamin D₃.

In vitro digestion was performed under subdued light to avoid vitamin D₃ degradation. The solutions and enzymes utilized during *in vitro* digestion were prepared as follows:

Gastric phase solution: (a) 0.9% saline solution (sodium chloride ACS, Fisher Scientific Company LLC., Hanover Park, IL.); (b) 4 g/L pepsin from porcine gastric mucosa (Sigma-Aldrich, INC., Milwaukee, WI.) solution in 0.1M Hydrochloric acid (HCL NF/FCC, Fisher Scientific Company LLC., Hanover Park, IL.); (c) 5M HCL solution to modify pH.

Intestinal phase solution: (a) Digestive enzyme cocktail including 2 g/L pancreatin from porcine pancreas (Sigma-Aldrich, INC., Milwaukee, WI.) and 12 g/L bile extract (Sigma-Aldrich, INC., Milwaukee, WI.) dissolved in 0.1M NaHCO₃ (Sigma-Aldrich, INC., Milwaukee, WI.); (b) 0.9M solution of NaHCO₃ to modify pH. (c) 2M NaOH (Sigma-Aldrich, INC., Milwaukee, WI.) to modify pH.

Samples of cheese were prepared by adding 2 drops of DI water per gram of cheese and then mixed with a mortar and pestle. Briefly, 5 g of sample were placed in a 50 mL conical centrifuge tube. 27 mL of 0.9% saline solution was added followed by 2 mL of gastric phase solution. pH was measured and adjusted to 2.0 with a few drops of 5M HCl solution. Samples were incubated with orbital shaking at 37 °C and 95 rpm for 1 h. Afterward, samples were placed in an ice water bath to decrease the enzymatic action. pH was adjusted to 5.3 by addition of few drops of 0.9M NaHCO₃ solution and 9 mL of intestine phase solution was added. The final pH was adjusted to 7.5 with few drops of 2M NaOH. Samples were incubated with orbital shaking at 37 °C and 95 rpm for 2 h. At the end of digestion, samples were centrifuged at 4000 g for 20 min at 10 °C. After centrifugation, samples were expected to show three phases or layers (sediment at the bottom, a clear micelle phase in the middle and a thin creamed phase at the top) (Figure 4.1); the bioaccessible fraction of liposoluble components is within the micellar phase (Ozturk and others 2015) since it is solubilized by the bile salts (Carbonell-Capella and others 2014). 4 mL aliquot was taken from the micellar phase for extraction of vitamin D₃ and placed in a 50 mL conical centrifuge tube. 10 mL hexane and 4 mL double deionized H₂O were added. This mixture was vortexed for 2 min, and centrifuged at 2500x g for 5 min at 20 °C. The supernatant was collected, and the extraction was performed twice. Supernatant was placed under nitrogen stream to evaporate the hexane and immediately reconstituted with 1 mL 100% MeOH mobile

phase. Sample was filtered through a 0.22 μm PTFE (Macherey-Nagel Inc., Bethlehem, PA. U.S.A.) and injected in the HPLC using the same parameters described previously for extraction vitamin D₃. The bioaccessibility was calculated as the amount of recovered vitamin D₃ after *in vitro* digestion compared with the amount of vitamin D₃ found in the cheese matrix extraction.

Statistical analysis

Statistical analysis was performed using Statistical Analysis System software (SAS[®] 2016, SAS Institute Inc., Cari, NC., U.S.A). Analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) were used to analyze statistical differences between samples (vitamin D₃ Extraction and recovery after *in vitro* digestion) with a type I error significance level (α) of 0.05.

Paired t-test (2-level treatments) was conducted to analyze the differences between cheese milk after microfluidization and after heat treatment since the same sample under different processing conditions was measured. Independent t-test (2-level treatments) was conducted to make comparisons between samples analyzed at different protein fat ratios and homogenization pressures processed at 17 MPa and 2 P/F ratio respectively.

4.4 Results and Discussion

Model fresh cheese fortification with vitamin D₃ and extraction

Three different samples of a model fresh cheese were selected from the experimental design explained in chapter 3 for vitamin D₃ fortification (Table 3.2) samples P1R1 (17 MPa – 0.9 P/F ratio), P1R4 (17 MPa – 2 P/F ratio) and P4R4 (150 MPa – 2 P/F ratio) to evaluate differences in vitamin D₃ bioaccessibility from the lowest to highest protein to fat ratios and

homogenization pressures respectively. Cheese milk was fortified with a stock solution of crystalline vitamin D₃ to a concentration of 2.5 µg/mL (Kazmi and others 2007; Wagner and others 2008), to compensate for the small sample size used in the extraction methods, so the amount of vitamin D₃ added was greater than concentrations regularly added to commercial food products. Vitamin D₃ concentration was recorded at different steps of cheese-making process, after homogenization, heat treatment, whey expelled during curd formation and the final cheese product (Table 4.1). For the cheese milk analyzed after microfluidization and after heat treatment, the amount of vitamin D₃ did not change significantly which means that vitamin D₃ was stable during the heat treatment. When comparisons were made between treatments, the cheese milk resulted in significant ($p < 0.05$) smaller levels of vitamin D₃ when processed at higher P/F ratios (2 P/F) and homogenization pressures (150 MPa) (Table 4.1). The main difference between the cheese milk samples was the particle size of the emulsion; samples processed at a higher protein to fat ratios and higher homogenization pressures resulted in a smaller particle size (results shown in chapter 3). Also, it has been mentioned, that the extraction of fat soluble vitamins might be influenced by their homogeneity within the matrix. This was theoretically explained by Blake 2007, who mentioned that encapsulated and more heterogeneously distributed compounds might affect the precision of the analytical procedure, and also, coating materials such as proteins may increase the difficulty of extraction of fat soluble vitamins. So, assuming that a significant amount of vitamin D₃ was accumulated within the matrix (since similar quantities were found in the model fresh cheese of the samples selected), a reduction in particle size along with a more stable interphase formed by caseins and whey proteins (Berton and others 2012; Farhang and Corredig 2012) might have hindered the ability to extract cholecalciferol by the method utilized.

As previously mentioned, after curd formation and pressing, similar ($p < 0.05$) amounts of vitamin D₃ were found for each treatment of cheese (Table 4.1). No vitamin D₃ was detected in the whey for samples processed at 17 MPa. However for the sample processed at 150 MPa, an amount below the limit of detection was identified. This difference may be due to the curd formation step in which higher homogenization pressured resulted in a smaller and more shattered curd, so an increased surface area may have caused trace quantities of vitamin D₃ to be expelled in the whey; however this need to be further investigated.

Bioaccessibility by *in vitro* digestion

Bioaccessibility was measured after *in vitro* digestion as the percentage of vitamin D₃ recovery in the micellar phase compared to the amount of vitamin D₃ found in the model fresh cheese.. Overall, samples processed at higher P/F (2 P/F) ratios and homogenization pressure (150 MPa) had lower vitamin D₃ recovery in the micellar phase (Figures 4.2 and 4.3). Given that vitamin D₃ is liposoluble, lower levels of fat could decrease the retention and stability of the compound in the matrix (Wagner and others 2008) and thus result in lower levels of vitamin after digestion. Liang and others (2010) studied β -lactoglobulin emulsion gels during *in vitro* digestion for the release of α -tocopherol, they concluded that a complete digestion is governed by the protein matrix degradation and partially hydrolyzed products of the β -lactoglobulin located at the oil/water interphase might prevent the release of α -tocopherol. The same hypothesis may apply to vitamin D₃, in which increasing P/F ratio and homogenization pressure could enhance the protein-protein interactions, so the time of enzymatic action during gastric digestion may take longer to efficiently degrade the protein matrix to obtain a higher rate of

micellarization and subsequent bioaccessibility. Similarly, Guo and others (2014) analyzed the behavior of whey protein emulsion gels during oral and gastric digestion concluding the release of oil droplets was faster in matrices containing large particle sizes due to coalescence of the oil droplets, compared to those at smaller particle sizes in which the destruction of the protein matrix was limited due to a thicker coating of the oil droplets. Simulated *in vitro* digestion are non-validated methods that may vary according to the matrix or compound under study, considering stronger interactions of casein aggregates in samples with higher protein to fat ratios and homogenization pressures, larger times of gastric digestion could improve the disruption of the casein matrix (Liang and others 2010).

Images of the protein and fat distribution of the model fresh cheese samples were taken by Confocal Laser Scanning Microscopy (Figure 4.4). The images showed that at a higher P/F ratios and homogenization pressures, there are smaller and more dispersed fat droplets within the protein matrix, strengthening the assumption that particle size is the factor affecting the release of vitamin D₃.

4.5 Conclusions

For this study, bioaccessibility of vitamin D₃ was analyzed by *in vitro* digestion in a model fresh cheese processed at different formulations and homogenization pressures. Increasing protein to fat ratio and homogenization pressure decreased vitamin D₃ extraction in cheese milk emulsion and bioaccessibility in a model fresh cheese. Given that the amount of vitamin D₃ resulted statistically similar between samples, the reduction in vitamin D₃ bioaccessibility might be related to the microstructural composition and behavior of the model cheese components at the interphase and coexistence between the two main phases (protein to fat) at the time of

digestion and extraction. In conclusion, changes in formulation and homogenization pressures had an effect on the structure of a model fresh cheese, although it was hypothesized that increasing the surface area of the cheese milk emulsion would lead to higher vitamin D₃ bioaccessibility, it was found that other factors such as the interfacial composition and the molecular interactions between the continuous phase (i.e. protein-protein interactions, or protein hydrolysis during digestion) could decrease micronutrient bioaccessibility.

4.6 Literature Cited

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4.7 Tables and Figures

Table 4.1 Vitamin D₃ recovery measured throughout processing and after *in vitro* digestion.

Sample	Cheese milk after microfluidization	Cheese milk after heat treatment (82 °C ~ 35 min)	In whey	Model fresh cheese	Vitamin D ₃ recovery after <i>in vitro</i> digestion
	µg/mL	µg/mL	µg/mL	µg/g	%
P1R1	1.89 ± 0.17 ^{a,*}	1.64 ± 0.03 ^{a,*}	N/D	4.88 ± 0.16 ^a	64.51 ± 9.96 ^a
P1R4	1.38 ± 0.11 ^{b,*}	1.40 ± 0.11 ^{b,*}	N/D	4.98 ± 0.28 ^a	41.56 ± 3.63 ^b
P4R4	0.98 ± 0.19 ^{c,*}	0.98 ± 0.17 ^{c,*}	0.31 ± 0.03	4.99 ± 0.31 ^a	27.17 ± 2.90 ^c

P1R1 = sample processed at 17 MPa and 0.9 protein to fat ratio.

P1R4 = sample processed at 17 MPa and 2 protein to fat ratio.

P4R4 = sample processed at 150 MPa and 2 protein to fat ratio.

N/D = Non detected

Averages of the vitamin D₃ recovery measured throughout processing and after *in vitro* digestion. Results are expressed as mean ± standard deviation (*n*=3). Different letter within each column denotes significant (*p* < 0.05) differences between treatments.

* denotes non-significant differences between rows in cheese milk after microfluidization and after heat treatment, analyzed by paired t-test.

Figure 4.1 Model fresh cheese digesta after *in vitro* analysis.

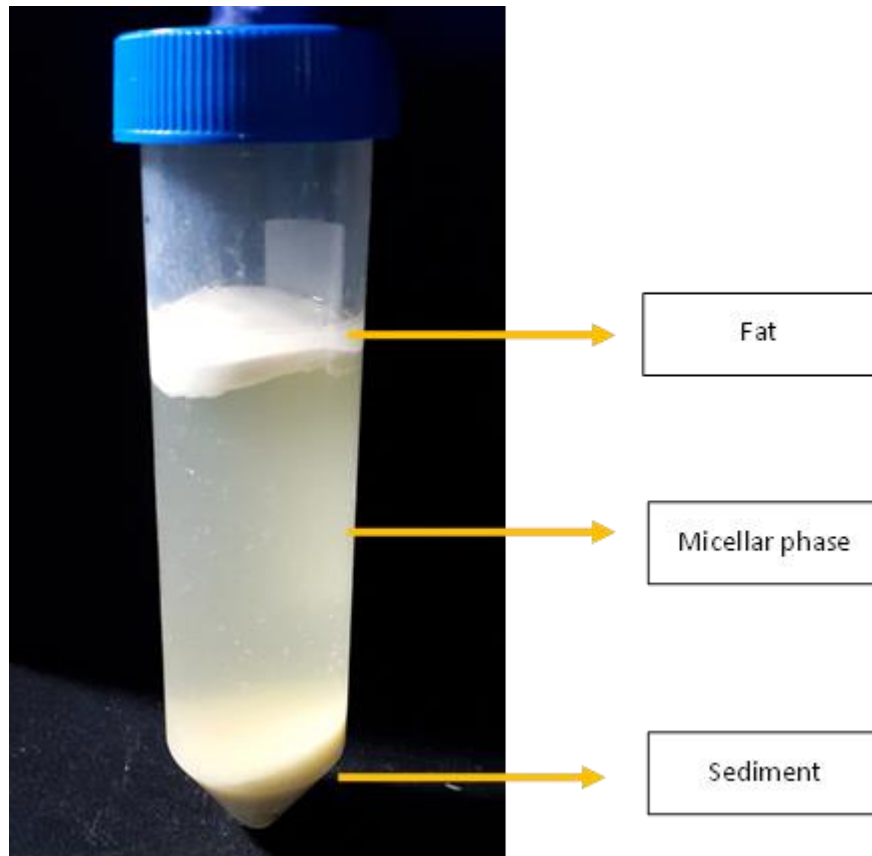
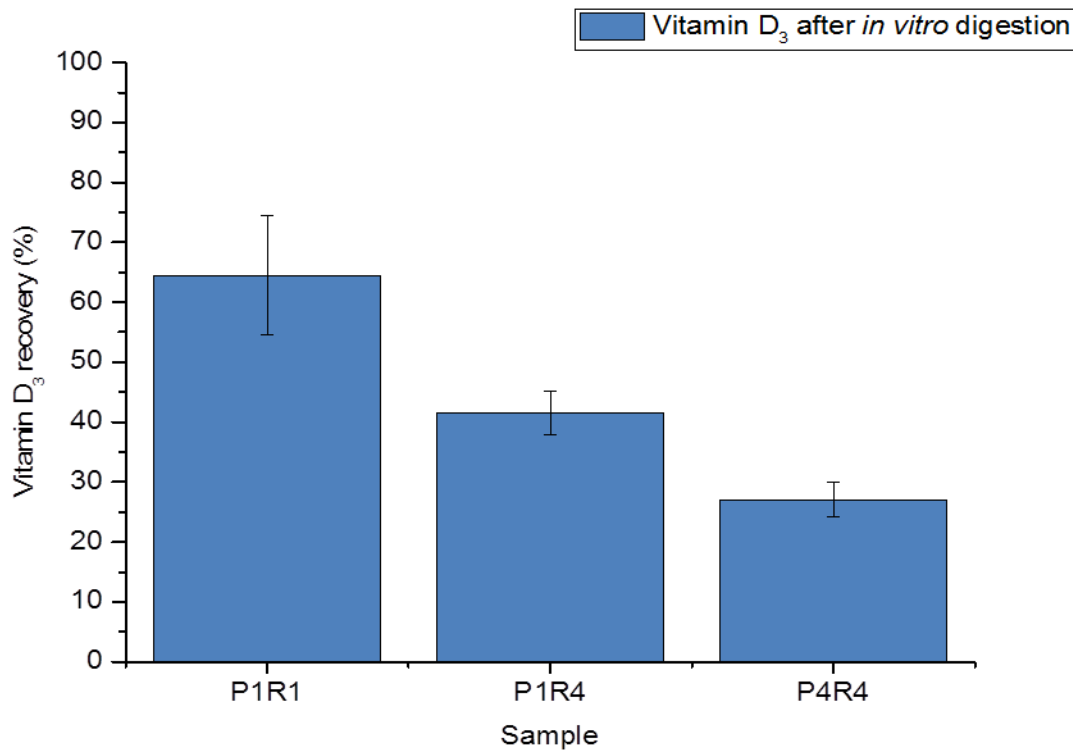


Figure 4.2 Bioaccessibility of vitamin D₃ of the fortified model fresh cheese



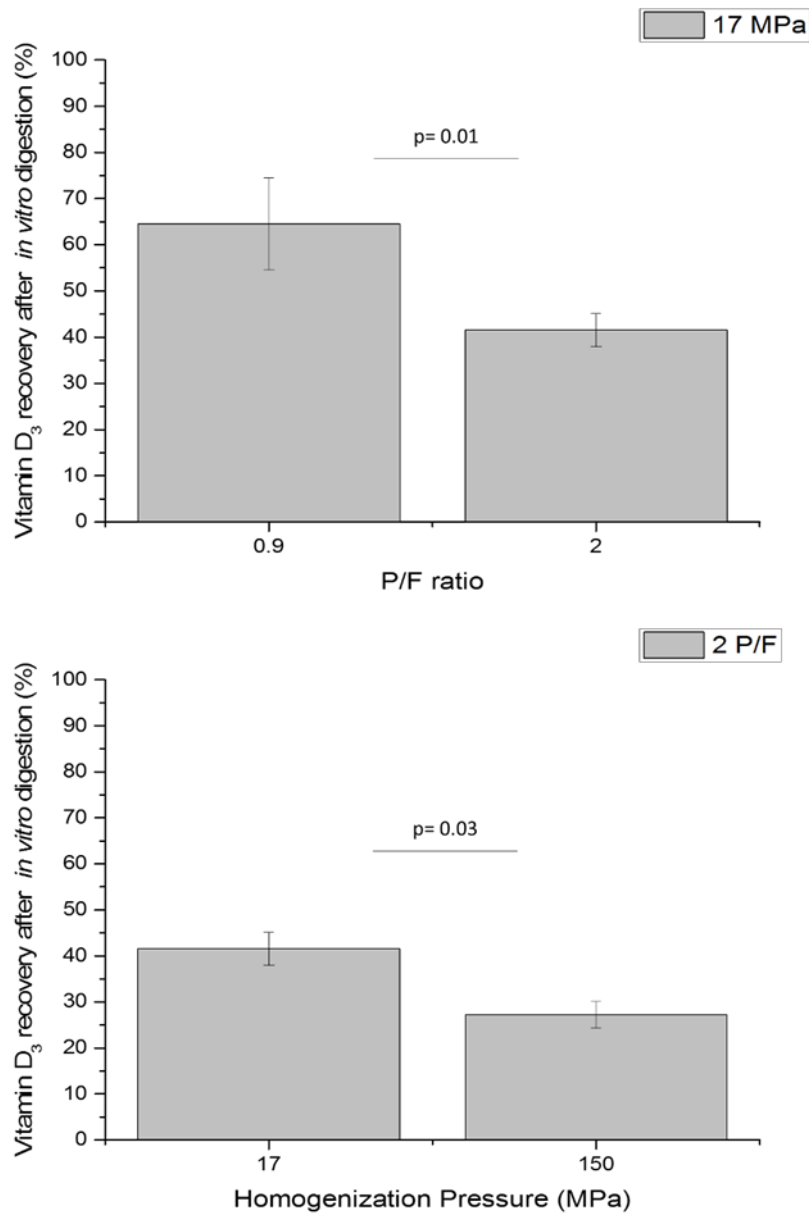
P1R1 = sample processed at 17 MPa and 0.9 protein to fat ratio.

P1R4 = sample processed at 17 MPa and 2 protein to fat ratio.

P4R4 = sample processed at 150 MPa and 2 protein to fat ratio.

Bioaccessibility as a percentage of the recovered vitamin D₃ in the micellar phase after *in vitro* digestion. Results expressed as mean \pm standard deviation (n=3). Different letter indicates statistical differences ($p < 0.05$) between treatments.

Figure 4.3 Effect of protein to fat ratio and homogenization pressure on vitamin D₃ bioaccessibility.



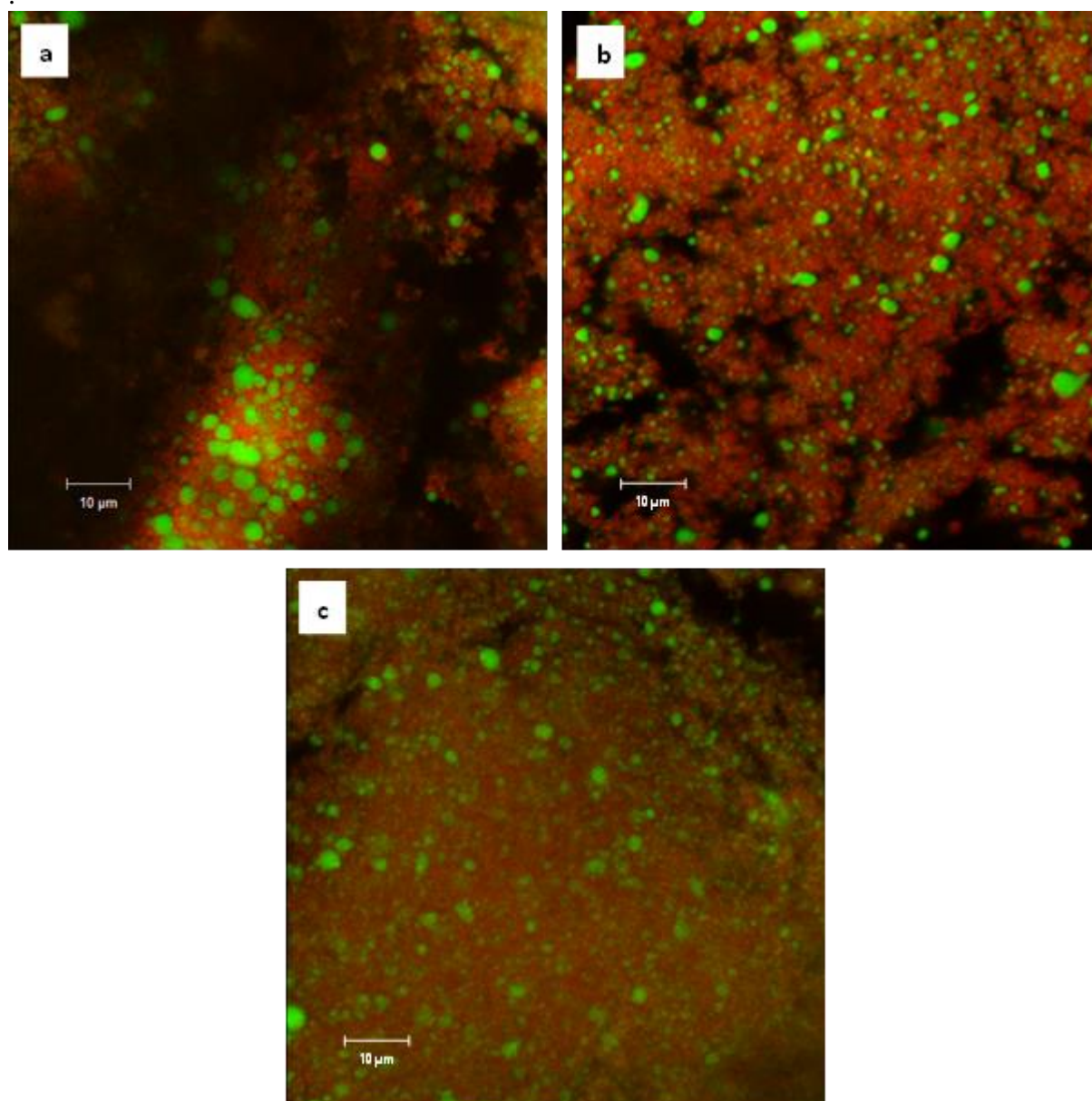
P1R1 = sample processed at 17 MPa and 0.9 protein to fat ratio.

P1R4 = sample processed at 17 MPa and 2 protein to fat ratio.

P1R4 = sample processed at 150 MPa and 2 protein to fat ratio.

Results expressed as mean \pm standard deviation (n=3) *p*-value was obtained by independent t-test, demonstrating significant differences between treatments

Figure 4.4 Confocal Laser Scanning Microscopy (CLSM) of the samples analyzed by *in vitro* digestion.



- a) P1R1 = sample processed at 17 MPa and 0.9 protein to fat ratio.
- b) P1R4 = sample processed at 17 MPa and 2 protein to fat ratio.
- c) P1R4 = sample processed at 150 MPa and 2 protein to fat ratio.

Chapter 5. Summary

In this study, the structure and microstructure of a model fresh cheese processed at various protein to fat ratios and homogenization pressures was evaluated and further analyzed for vitamin D₃ bioaccessibility. The overall conclusion is that diverse formulations and processing parameters contributed to modify the structure in a model fresh cheese affecting the bioaccessibility of vitamin D₃ during in vitro digestion. This finding suggests that understanding complex food matrices from a microstructural perspective might allow to the development of new products with specific functional characteristics improve the nutritional information provided to the consumer.

Model fresh cheeses were prepared by reconstituting skim milk powder and anhydrous milk fat in DI water at different levels of protein to fat ratios and homogenization pressures. Cheese curd was formed by induced acid coagulation and pressed overnight. The structural characteristics were measured at large and small deformations, using Texture Profile Analysis and Small Amplitude Oscillatory Shear rheometry. Particle size of the cheese milk emulsion was also analyzed by laser diffraction, and microstructure was assessed by Environmental Scanning Electron Microscopy (ESEM). Samples were fortified with vitamin D₃ to carry out the bioaccessibility study by a two phase in vitro digestion.

Structural parameters were predominantly affected by protein fat ratio than homogenization pressures, however both variables significantly affected vitamin D₃ bioaccessibility. At higher protein to fat ratios and homogenization pressures, vitamin D₃ bioaccessibility was decreased, suggesting higher interconnectivity of the protein matrix due to a major volume fraction of

protein compared to fat and higher shear forces decreasing the fat globule size, and thus leading to lower hydrolysis during digestion impeding the release of the fat soluble components.

Further research is needed to explore the interactions of food components in complex matrices, in order to precisely determining the critical factors (i.e. specific processing application or interphase kinetics) that contributes intrinsically to the structure formation, digestion and bioavailability of macro and micronutrients.